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Blood Coagulation and Lipid Metabolism

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BLOOD coagulation is the result of a complex series of reactions. Normally, once initiated, the whole process proceeds at an accelerating rate until completed. By the use of appropriate reagents, however, the process can be stopped rather easily at certain definite points, and it is common practice to discuss blood coagulation as though it takes place in several stages.

FIRST STAGE

The formation of thromboplastin can be considered the first stage. While there is no unanimity of opinion regarding the order in which the various plasma factors react to form thromboplastin, there is general agreement as to the importance of these factors. At least three bleeding conditions have been observed in which the common defect is in the formation of plasma thromboplastin. Each appears to be due to the lack of (or inactivation of) specific proteins present only in minute amounts. Since any hypothesis of blood coagulation must take these facts into account, a suggested scheme for the events in the first stage of coagulation is shown in Chart I antihemophilic factor (AHF), plasma thromboplastin component (PTC) and possibly other proteins (Stuart Factor, Hageman Factor, PTA) react with a lipid material from platelets to form thromboplastic activity. The reaction can be initiated by contact with a "wettable surface" or inflamed or injured area. This suggests that lysis of platelets may be the initiating factor. MacFarlane[1] gives evidence which suggests that PTC and AHF join with calcium ions to form an intermediate product which then combines with platelets.

AHF is present in normal fresh plasma but is absent or inactive in normal serum. It is not readily adsorbed on the usual prothrombin-adsorbing reagents. PTC is present in both normal plasma and serum and is readily adsorbed on prothrombin-adsorbing reagents. According to the work of Tocantins and Carroll[2] a thromboplastic inhibitor exists in plasma. They have isolated this material from both normal and hemophilic plasma. It appears to inhibit the development of thromboplastin as well as inactive thromboplastin after the latter has been formed. Thromboplastin can be obtained from tissues but this material appears to need factors from plasma and serum to make it fully active. Heparin inhibits the formation of thromboplastin[3] and the action of thromboplastin itself.

SECOND STAGE

The conversion of prothrombin to thrombin normally depends on the presence of thromboplastin and thus can be considered the second stage. The work of several laboratories has

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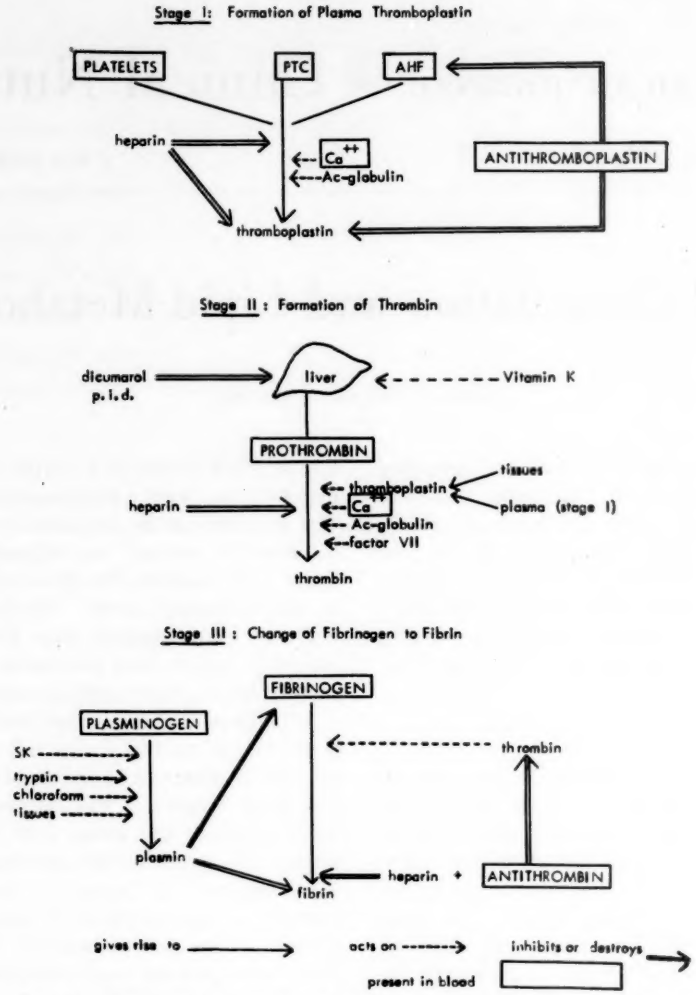

Chart 1. Schematic representation of the various reaction involved in the coagulation of blood. SK = streptokinase.

indicated the existence of certain plasma and serum factors which accelerate the conversion of prothrombin to thrombin. The plasma factor was discovered independently in widely separated laboratories and has been referred to by a variety of names. It appears to be associated with the globulin fraction of plasma and is normally present in only trace amounts.

Ware, Fahey and Seegers[4] found that the activity of this plasma AC-globulin was greatly increased during coagulation and that plasma AC-globulin fractions were made more potent by small amounts of thrombin. It is now generally accepted that serum contains material not normally active in plasma. The active material is readily adsorbed on prothrombin adsorbing reagents and is more stable than the plasma fraction. The term "factor VII" is most commonly used to designate the serum-active factor (SPCA, proconvertin, autoprothrombin are other names for this factor).

Alkjaersig, Johnson and Seegers[5] have shown that a derivative of purified prothrombin acts as an accelerator and have suggested that the

serum factor is derived from prothrombin during the coagulation process. In support of this hypothesis, it may be pointed out that during the administration of Dicumarol or Tromexan both prothrombin and factor VII levels are decreased, with factor VII often being the first to show change.

THIRD STAGE

Once thrombin is formed it acts enzymatically to convert soluble fibrinogen to insoluble fibrin. This reaction can be considered the third stage. Associated with events in the third stage of coagulation is the activation of plasminogen to plasmin and the subsequent lysis of fibrinogen and fibrin. This area has become so specialized and complex that it is often considered as a separate field, and will not be discussed in detail at the present time. One point, however, is relevant to the present discussion. It has been postulated that plasmin may function normally to prevent excess deposition of fibrin on the endothelium of the vessels. Greig and Runde[6] reported that plasmin is inhibited in the presence of fat and suggest that this inhibition might be a contributing factor to the development of atherosclerosis.

Plasma and serum contain a substance known as antithrombin, the physiological importance of which has not yet been determined. Attempts to correlate decrease in antithrombin with tendency toward thrombosis have been unsuccessful. Antithrombin was originally believed to be an albumin but recent work indicates that it is associated with the alpha globulins[7] Fig. 1.

The development of a synthetic substrate for thrombin[8] P-toluenesulfonyl-arginine-methyl ester (TAMe) has made it easier to distinguish between inhibition of thrombin and destruction of thrombin. Heparin-cofactor and antithrombin activities appear to be properties either of the same substance or of two closely related substances. It has so far been impossible to separate the two activities. Whenever one activity is concentrated the other is concentrated; destruction of one is associated with destruction of the other. As shown in Figure 2 heparin added to blood

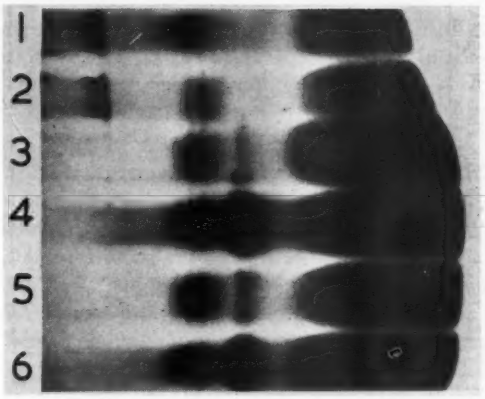

FIG. 1. Electrophoretic patterns on starch; 1, defibrinated plasma; 2, after adsorption with aluminum hydroxide; 3 and 5, fractions from citrate eluates; 4 and 6, fractions from phosphate eluates. Sample 2 had no antithrombin potency. Samples 4 and 6 had double the activity of samples 3 and 5.

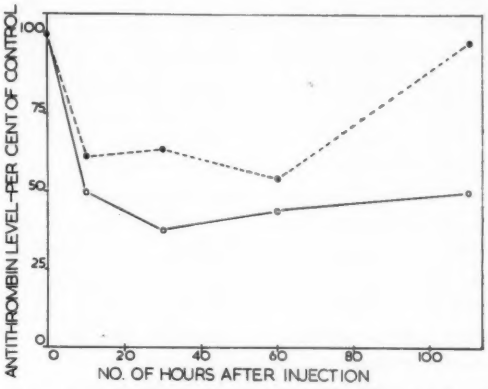

FIG. 2. The decrease in antithrombin level of plasma following an intravenous injection of heparin. ○—○ 500 units per kilo; ●—● 200 units per kilo.

in vitro, or injected intravenously, decreases the antithrombin titer. This may seem paradoxical. However, while the capacity of heparinized blood to destroy thrombin is reduced from normal the total thrombin *inhibitory* effect is greatly increased. Heparin achieves its inhibitory action by interfering with the thrombin-fibrinogen reaction, not by destroying thrombin.

ROLE OF FATS

There is accumulating evidence that the consumption of excessive amounts of fat or

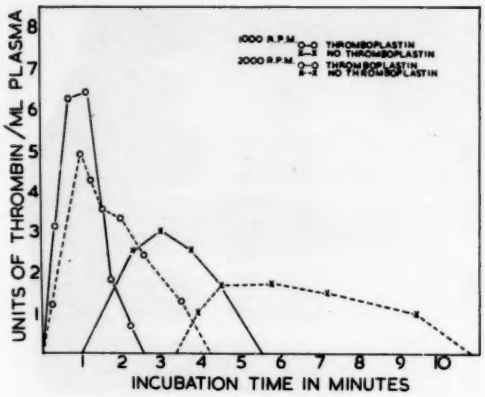

FIG. 3. The effect of rate of centrifugation and addition of thromboplastin on the thrombin generation of recalcified citrated dog plasma.

impairment in fat metabolism are factors in the development of atherosclerosis and thrombosis. The assumption, valid or not, that there is a relationship between blood clotting and thrombosis has led to an increasing number of studies on the role of fat in blood coagulation.

The results of tests carried out on whole blood have been controversial. Waldren, Beidelman and Duncan[9] found a shortening of the clotting time in siliconed tubes following a fat meal. Manning and Walford,[10] in similar experiments, failed to find any significant change. Merskey and Nossel[11] found that their results varied depending on the type of meal ingested.

More uniform results have been obtained when the tests have been carried out on plasma. Robinson *et al.*[12] found there was an increase in the concentration of free fatty acids in plasma following a fat meal. This increase is within the range where Poole[13] and Pilkington[14] demonstrated an *in vitro* effect with a variety of added fatty acids. Thus, some of the increase in coagulability may be the result of an increase in fatty acid concentration and the variability in results may be a consequence of the type of fatty acid adsorbed.

More emphasis however has been placed on the role of phospholipids in blood coagulation. O'Brien[15] observed that when the platelets are able to act there is no detectable difference in the clotting time of lipemic blood after a meal of fat compared with that from the same patient fasted. When platelets are removed or unable to act, lipemic blood clots more rapidly than the equivalent fasting sample. Thus an extremely important point to consider is the method of preparing the plasma. Pitney and Dacie[16] showed that the amount of thrombin generated when plasma is recalcified is influenced by the degree of centrifugation. This has been confirmed in our laboratory. Figure 3 illustrates the effect of variation in rate of centrifugation on the thrombin generation of recalcified citrated plasma.

O'Brien[15] introduced what is known as the "Stypven®" time. He showed that the addition of Russell viper venom to plasma caused a greater decrease in the clotting time of citrated plasma four hours after a fat meal than after fasting. This has been confirmed by others.[17] As early as 1941 Macfarlane, Treven and Atwood[18] showed that crude lecithin, chylomicra or milk-fat augmented the thromboplastic activity of Russell viper venom. Merskey and Nossel[11] showed that the decrease in "Stypven" time was independent of the type of fat ingested. Thus the "Stypven" time may merely reflect the degree of lipemia and be unrelated to any real change in the coagulability of blood.

Poole[19] found that when plasma was freed from chylomicra by high speed centrifugation its clotting time was increased. The clotting time of such plasma could be returned to normal by the addition of a washed suspension of chylomicra. With Robinson[20] Poole showed that the active fraction of chylomicra was phosphatidyl ethanolamine, and later[21] that the clotting time of high-spun plasma could be greatly decreased by adding phosphatidyl ethanolamine to it. Since the lack of platelets is the most noticeable characteristic of high-spun plasma it was logical to suppose that the thromboplastic activity of platelets might be due to their phosphatidyl ethanolamine content. Bell and Alton[22] used a chloroform extract of acetone dried brain and O'Brien[23] a phosphatide fraction from eggs as substitute for platelets in the thromboplastin generation tests. Thus it has been fairly generally assumed that the activity

of platelets is due to their content of phosphatidyl ethanolamine. Recently however Spaet and Marcus[24] and Troup and Reed[25] have fractionated lipid extracts of platelets and red cells and claim that phosphatidyl serine is much more active in the formation of thromboplastin than phosphatidyl ethanolamine. Thus, there is still some question regarding the exact identity of the active platelet lipid. The answer awaits the development of more accurate methods of identification.

ANTICOAGULATION

While we are concerned with the coagulant properties of lipids we should not completely ignore their anticoagulant properties. During the past ten years Tocantins and his colleagues have carried out an extensive study on a lipid anticoagulant fraction from brain. They have succeeded in obtaining a highly purified substance which, on the basis of chemical tests, has been identified as phosphatidyl serine.[26] The extract is active both *in vitro* and *in vivo*. The anticoagulant activity of their phosphatidyl serine from brain appears to be in direct contrast to the coagulant activity of the phosphatidyl serine fraction prepared from platelets by others.[24] Recently however Barkhan and associates[27] demonstrated that phosphatidyl serine from brain can act *in vitro* either as an accelerator or as an inhibitor of thromboplastin formation depending on experimental conditions. Thromboplastin formation was inhibited when the phosphatidyl serine in aqueous solution was placed in the reaction mixture. When the phosphatidyl serine was first suspended in plasma which had been decalcified by ethylene diamine tetraacetic acid (EDTA) and adsorbed by aluminum hydroxide, and then added to the reaction mixture thromboplastic formation was enhanced. This potentiating effect was not found when citrated or oxalated plasma was used. Thus the same substance may exhibit either coagulant or anticoagulant characteristics *in vitro*, depending on the concentration of the substance and the type of test used. Further work may reveal that the clotting activities of the various phosphatides depend upon the type, and degree of saturation of the fatty acids incorporated in the molecule.

CONCLUSIONS

It is reasonable to conclude that lipids, especially the phosphatides, play an important part in the reactions which result in the formation of a blood clot. Not all lipids are coagulants. Changes in the level of fatty acids and phosphatides result in changes in a number of *in vitro* coagulation tests. However, variations in the same coagulation test can be demonstrated simply by varying the rate of centrifugation, the type of decalcifying agents, or even the order in which the reagents are added. Thus at present we have no clear understanding how changes in static blood are related to the development of an intravascular clot. It is unlikely that any changes in the coagulability of blood brought about by variations in the lipid level are important in the presence of a healthy endothelium or a normal blood flow rate. Such changes, however, may be significant when superimposed on degenerative changes in the vessel wall or when circulation is impaired. The clinical importance of lipids on blood clotting can only be ascertained when there is a better knowledge of the relationship between blood clotting and vascular changes.

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The Heparin Clearing Reaction

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I WOULD like to discuss our present knowledge of the heparin clearing reaction and to make one or two observations regarding its possible role in fat transport.

THE NATURE OF THE HEPARIN CLEARING REACTION

During the absorption of a fatty meal there is frequently an alimentary lipaemia due to the presence of lipid particles in the plasma which are composed primarily of triglyceride and which are derived directly from the chylomicrons in the thoracic duct. The alimentary lipaemia clears rapidly following the intravenous injection of heparin. We understand this to be the heparin clearing reaction *in vivo*.

If a plasma sample is withdrawn from a person who is not consuming a fatty meal but who has been given an injection of heparin (such plasma will be referred to as heparinized plasma), this plasma will have the ability to clear *in vitro* the turbidity of an added particulate lipid emulsion. This is known as the heparin clearing reaction *in vitro*. The addition of heparin to normal blood *in vitro* does not give plasma obtained from this blood the ability to clear an added lipid emulsion. Active plasma can only be obtained after the intravenous injection of heparin and the possibility that heparin activates a substance already present in the blood is excluded.

Lipid preparations which have been used in the studies *in vitro* include turbid lipid particles recovered from lipemic plasma, artificial triglyceride emulsions and chyle obtained by cannulation of the thoracic ducts of animals which have been fed a fatty meal.

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It is generally accepted that clearing of a lipid emulsion by heparinized plasma *in vitro* occurs because the injection of heparin induces a lipase in the plasma which breaks down the triglyceride component of the emulsion liberating unesterified fatty acids which go into solution with the plasma albumin.[1] The result *in vitro* is a disappearance of triglyceride, an appearance of unesterified fatty acids in combination with the plasma albumin and, because the triglyceride was in a turbid lipid system and the unesterified fatty acids are in a water-soluble state, a clearing of the lipid emulsion.[2] There is no change in the total fatty acid content of such a system.

When heparin is injected *in vivo* during the absorption of a fatty meal, the changes which occur in the plasma lipids appear to be explained adequately by the assumption that the same lipolytic system active *in vitro* is active *in vivo*. Thus, *in vivo* there is a drop in the concentration of the low density lipoproteins with a high triglyceride content and a parallel rise in the concentration of the denser components containing less triglyceride.[3–5] Normally there is a small amount of unesterified fatty acids in plasma combined with the plasma albumin. This amount, as might be expected when intravascular lipolysis occurs, is raised somewhat after the injection of heparin during alimentary lipaemia.[2] Paper electrophoretic studies carried out on plasma samples obtained after the injection of heparin during alimentary lipaemia show that the chylomicron component of the plasma is reduced in size and the mobility of the high and low density lipoproteins is increased.[6–8] An association with lipoproteins of a small proportion of the unesterified fatty acids released by lipase action, as observed *in vitro*, causes such an increase in the mobility of lipoprotein.[9–11]

In addition to the release of lipase in the

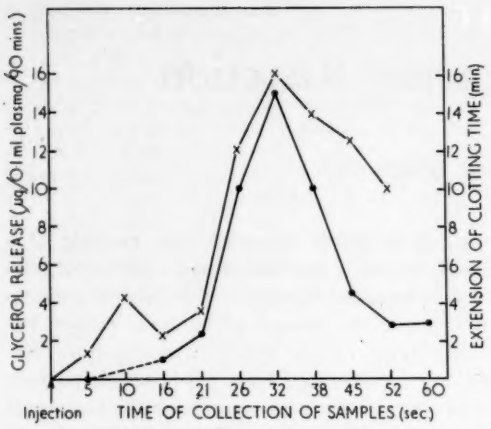

FIG. 1. The lipolytic and anticoagulant activity of plasma samples collected from the femoral vein of the rabbit after the injection of heparin into the femoral artery. Successive samples of blood (0.4 ml.) were collected from the femoral vein after the injection of 0.1 ml. of heparin (1,000 units per ml.). The lipase activity of the plasma separated from each blood sample was determined at 37° C. by incubating 0.12 ml. of each plasma sample with 0.14 ml. of a 10 per cent solution of bovine albumin in water at pH 8.2 and 0.014 ml. of chyle. The glycerol content of triplicate aliquots (0.05 ml.) of this mixture was determined after zero and ninety minutes ($\bar{x}$). The anticoagulant activity of the plasma samples was measured in terms of the extension of the clotting time of normal rat plasma (●). From: ROBINSON, D. S. and HARRIS, P. M. The production of lipolytic activity in the circulation of the hind limb in response to heparin. *Quart. J. Exper. Physiol.*, 44: 80, 1959.

plasma after the injection of heparin, the possibility exists that the lipolytic changes which result *in vivo* will affect the rate at which the dietary fat leaves the circulation. The injection of heparin has been shown to accelerate the removal of ingested or injected chylomicron triglyceride from the blood.[12–14] Apparently, this is because of the rapid disappearance of the unesterified fatty acids (the products of lipolytic action) from the circulation. While the circulating half-life of injected triglyceride, in the absence of any heparin-induced intravascular lipolysis, is about ten minutes (varying somewhat with the amount of triglyceride injected),[14–15] the half-life of injected unesterified fatty acids is only one or two minutes.[15,16]

In summary then, the effect of an injection of heparin *in vivo* in clearing an alimentary lipaemia can be explained in terms of its release of the clearing factor lipase into the blood with resultant intravascular lipolysis of triglyceride and the rapid removal of the products of lipolysis (the unesterified fatty acid) from the circulation.

The clearing factor lipase only hydrolyses triglycerides and has no direct action on phosphatides and cholesterol esters.[17] It has been found, however, that a sample of heparinized plasma, which has already cleared chyle *in vitro*, has the ability to clear the turbidity of an added cholesterol suspension.[18,19] The extent of this secondary clearing of cholesterol is dependent on the amount of chyle already cleared and hence on the amount of unesterified fatty acids released. The process appears to be analogous to the increase in the solubility of cholesterol which occurs in soap solutions, and it seems that some type of cholesterol-unesterified fatty acids-protein complex may be formed in the plasma. This is evidently an artificial *in vitro* system; however, it is possible that the redistribution of cholesterol which occurs *in vivo* during the clearing (by the heparin-induced lipase) of a particulate lipid preparation containing cholesterol as well as triglyceride, may be due to a similar association of cholesterol with the released unesterified fatty acids.[5]

SOME PROPERTIES OF THE HEPARIN CLEARING REACTION

Extremely small amounts of heparin are required to induce lipolytic activity in the plasma, e.g., the injection of 1 μg. of heparin/kg. of body weight in the rat.[20] The actual level of activity which is produced in the plasma, in any experiment, depends upon the amount of heparin injected, up to a maximum, which itself depends on the species and on the interval between injection and withdrawal of blood.[20–22]

The appearance of lipolytic activity in the plasma is extremely rapid after the injection of heparin.[23] The results of an experiment in which heparin was injected into the femoral artery of the hind limb of a rabbit, whose femoral vein was cannulated, are shown in Figure 1. Beginning just before and continu-

ing throughout the injection all the venous blood was collected from the femoral vein in a series of samples. The injection of heparin into the blood circulating through the hind limb was completed in about half a second, and the appearance of this injected heparin in the venous blood was detected by its anticoagulant effect. It was found that the first sample of blood to contain heparin, collected about twenty seconds after the injection, also possessed lipolytic activity. There was *no lag period* before the lipase could be detected. In other similar experiments, in which Evans blue dye was injected with the heparin, lipolytic activity appeared in the same sample of venous blood as did the dye.

This immediate appearance of lipase in the blood following the injection of heparin suggests that the enzyme must be released from some site immediately accessible to substances in the circulating blood, perhaps from the wall of the blood vessel.

Supporting evidence for this view comes from experiments in which a series of dextran sulphate preparations, all of similar high sulphur content but of varying molecular weight, have been used.[24] When injected, dextran sulphate, like heparin and also other charged molecules, such as sulphated cellulose, sulphated hyaluronic acid, sulphated alginic acid and phosphomolybdate, phosphotungstate and silicotungstate, releases the clearing factor lipase into the blood.[25] The molecular weight range of each preparation which was injected was narrow but the molecular weights of the different preparations varied from about a thousand to several million. It was found, in preliminary experiments, that the high molecular weight dextran sulphates, like high concentrations of heparin, inhibited the clearing factor lipase which they induced, but that this inhibition could be reversed in the presence of protamine sulphate. Testing for lipolytic activity was, therefore, carried out in the presence of protamine sulphate.

When the dextran sulphates were injected into rats, lipolytic activity was produced in the blood within thirty seconds by the whole range of preparations. Results with two of these preparations of the highest molecular

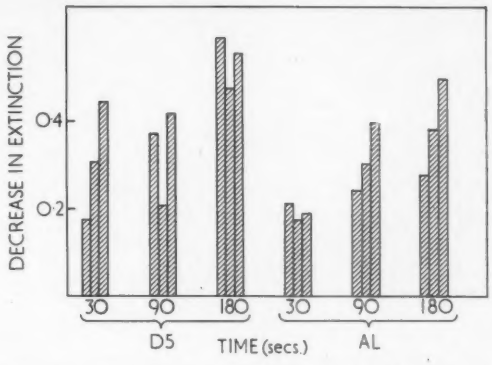

FIG. 2. The clearing activity induced by the injection of 250 μg. of the dextran sulphate preparations D5 and AL. Groups of rats were bled thirty, ninety or 180 seconds after injection. Each plasma (0.4 ml.) was mixed at 0° C. with 0.2 ml. of 0.05 per cent protamine solution and 0.3 ml. of 10 per cent albumin solution in water (pH 8.2). After ten minutes 20 μl. of chyle was added and, after a further five minutes at 0° C., the decrease in the extinction of each system was followed at 37° C. The height of each column represents the decrease in the extinction in thirty minutes of an individual plasma system. (From: ROBINSON, D. S., HARRIS, P. M. and RICKETTS, C. R. The production of lipolytic activity in rat plasma after the intravenous injection of dextran sulphate. *Biochem. J.*, 71: 286, 1959.)

weight (D5, 2×10^5; AL, $>2 \times 10^6$) are shown in Figure 2.

Although the response was markedly less than that which occurred with an equivalent amount of heparin, the rapid appearance of the lipolytic activity in the blood would suggest that dextran sulphates of such large molecular weights release the lipase into the circulation without leaving the blood stream themselves.

Although the clearing factor lipase is not produced when blood and heparin are mixed *in vitro* it is formed when heparin and blood are perfused through restricted parts of the circulation, as shown by the experiments in the rabbit. Further perfusion experiments have been carried out in the rat.[23] In these experiments isolated rat hind limb preparations were first perfused with albumin solution so as to remove blood from the vessels. Then individual preparations were perfused with either heparin in serum, or heparin in citrated plasma or heparin in 5 per cent albumin in

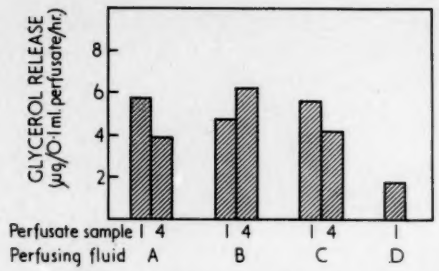

FIG. 3. The lipolytic activity of the perfusates after the perfusion of various mixtures containing heparin (0.2 unit per ml.) through the preparation of the hind limb of the rat. Preparations were perfused with heparin in 10 ml. of either (A) citrated plasma; (B) serum; or (C) 5 per cent bovine albumin in Hank's solution at pH 7.4. One preparation (D) was perfused with citrated plasma alone. In each case the perfusate was collected in four successive samples of 2.5 ml. each. The lipolytic activity of the first and last samples was measured by incubating, at 37° C., 0.5 ml. of the sample with 0.5 ml. of a 10 per cent solution of bovine albumin in water at pH 8.2 and 0.03 ml. of chyle. The glycerol content of triplicate aliquots (0.05 ml.) of this mixture was determined after zero and seventy-five minutes. (From: ROBINSON, D. S. and HARRIS, P. M. The production of lipolytic activity in the circulation of the hind limb in response to heparin. *Quart. J. Exper. Physiol.*, 44: 80, 1959.)

Hank's solution. In all cases the initial perfusate sample was found to contain lipolytic activity and this activity persisted at a high level in later samples of the perfusate. This is shown in Figure 3. When heparin in plasma was perfused again through the hind limb preparation the lipolytic activity in the perfusate was augmented. The perfusion of citrated plasma alone released some activity which was enhanced by the addition of heparin *in vitro*. However, the level of activity was variable and further investigation of the point is needed.

It appears from these experiments that, no plasma factor is involved in the production and action of the clearing factor lipase other than the plasma albumin in the role of acceptor of the unesterified fatty acids (c.f., 36). However, in our study, it was not possible to remove all the blood from the perfused limb by the perfusion of albumin alone and the perfusate, after the passage of heparin and albumin through the limb, was always contaminated with red cells and presumably plasma proteins. It is still conceivable that a small amount of some plasma protein other than the plasma albumin is required.

These results, taken together, suggest that the clearing factor lipase may be sited in the walls of the blood vessel. Such a conclusion does not conflict with the presence of clearing factor lipase in extracts of heart tissue[26,27] and of adipose tissue[28] since these studies do not indicate the site of the enzyme in these tissues. The presence of the enzyme, either specifically in association with the blood vessels of heart and adipose tissue, or generally in walls of the blood vessels throughout the body, might explain both groups of observations. Alternatively, the enzyme may be present intracellularly in certain tissues as well as in association with the blood vessels.

There is some independent evidence that heparin may have an effect on a constituent of the inner surface of the wall of the blood vessel. Thus, when the inner surface of an artery or vein is exposed to heparin and then to toluidine blue or a similar basic dye, the dye is bound at the surface of the endothelial cells.[29,30] When the artery or vein is exposed to toluidine blue alone the dye is not bound. The observations of Zemplényi and Grafnetter,[31] that minced aortic tissue possesses lipolytic activity, are also of considerable interest in this connection.

It appears that heparin plays an integral part in the action of the clearing factor lipase and may stabilize the enzyme. Thus, a bacterial heparinase has been shown to destroy the lipase activity[32] and, after the passage of active heparinized plasma down an anion exchange column which removes at least part of the heparin, the effluent from the column, although it has full activity initially, loses this activity on incubation for a short time at 37° C.[33] In the presence of added heparin, the activity of the effluent is maintained at 37° C.

The apparent inhibition of the action of the clearing factor lipase by protamine sulphate and certain basic dyes[34–37] may be due to the ability of these substances to combine with heparin and so reduce the stability of the

enzyme system. Thus, in the presence of protamine sulphate, the activity of a sample of heparinized plasma *in vitro* is high if measured immediately, but is lost after incubation at 37° C. for ten minutes.[24]

When the clearing factor lipase acts on chylomicron triglyceride a combination of enzyme and substrate can be demonstrated.[20] Thus, if chyle and heparinized plasma are mixed at 0° C., and then centrifuged at 0° C., the infranatant plasma loses most of its activity which becomes associated with the floating layer of chyle. Such close association is presumably essential if a water-soluble lipase is to act on a triglyceride substrate present in a second oil phase.

It is possible that heparin may act as the link between the water-soluble lipase and its lipid substrate, and that the inhibition of the lipase activity by high concentrations of salt and by pyrophosphate is due to the displacement of this bound heparin.[26] The inhibitory action of the high molecular weight dextran sulphates and of high concentrations of heparin may also be due to the ability of these molecules to occupy the sites on the lipid substrate which are normally the points of attachment for the heparin-lipase complex.

The studies of Korn[27] and of Korn and Quigley[38] on the clearing factor lipase activity of extracts of heart and adipose tissue led them to suggest that a triglyceride substrate had to be associated with lipoprotein material before it could be attacked. They suggested the term lipoprotein lipase for the clearing factor lipase. Their observation has been confirmed by others, although, in later studies, Korn and Quigley observed that under certain conditions up to 50 per cent of the maximal lipolytic activity could be demonstrated with lipid emulsions which did not contain added lipoprotein.[28]

Chylomicra, as they are recovered from the thoracic duct during fat absorption, appear to contain small amounts of protein and are adequate substrates for the clearing factor lipase.[27,39–41] In our own studies[42] this protein component was not detected in chylomicron preparations in which the subnatant lymph proteins had been removed by a process of repeated centrifugation, separation and resuspension. Subsequent workers, however, have found between 0.2 and 2 per cent associated protein, the amount varying with the particular preparation studied. It appears that the amount of protein in such preparations rises as the speed of centrifugation used in the washing procedure is increased. It is possible that in using high speeds of centrifugation some of the preparations analysed may have been contaminated with low density lipoprotein material present in the lymph. This would raise the apparent protein content of the chylomicron by the inclusion of material which was not a true constituent of that fraction, but rather a constituent of the subnatant lymph. Using lower rates of centrifugation, comparable to those in our own earlier studies,[41] Bragdon has reported a protein content for a chylomicron preparation of only 0.2 per cent, and suggests that some chylomicra may contain no protein at all. The stability of the chylomicron emulsion seems to depend primarily on its phosphatide content: the amount of protein present appears to be insufficient to cover the entire surface of the lipid particles.

Some recent studies on the protein in chylomicra from lymph suggest that it is heterogeneous and that one component is identical with one of the high density lipoprotein fractions of the plasma.[43]

Following their entry into the blood the chylomicra become associated with more protein material than they contain when in the thoracic duct.[42,42a]

THE ROLE OF THE CLEARING FACTOR LIPASE

With this knowledge of the properties of the clearing factor lipase in mind we can consider the question of the possible physiologic role of the enzyme system in fat transport. Is there evidence that it is involved in the transport of chylomicron triglyceride from the blood to the tissues, or in the mobilization of the depot lipids, or in both these processes?

Removal of Chylomicron Triglyceride from the Blood

Dietary triglyceride, which enters the blood from the thoracic duct in lipid particles, the

chylomicra, some 0.5 to 1.5 μ in diameter, leaves the circulation again extremely rapidly. Thus, in the rat, after a fatty meal, chylomicron triglyceride enters the blood at the rate of about 1 mg. per minute, but the sustained rise in the triglyceride content of the plasma during alimentary lipaemia is small (from a total triglyceride content of about 5 mg. to one of 7 mg.),[44] and it is evident, that the rate of removal of the chylomicron triglyceride from the blood is equivalent to its rate of entry. Again, in the experiments already mentioned in which the rate of removal from the circulation of injected chylomicron triglyceride was studied, it was shown that after the injection of about 10 mg. of triglyceride, the circulating half-life of the triglyceride was only six minutes.[14,15]

These rates of removal of chylomicron triglyceride are much faster than the rates of removal of lipoprotein phosphatide and cholesterol and pose the problem of the mechanism of removal from the blood of triglyceride present in particles of the size of the chylomicrons. Two possibilities can be considered: either the chylomicrons leave the circulation unchanged as particles, or they are degraded in some way into a more readily diffusible form before they cross the blood vessel walls: lipolysis of the chylomicron triglyceride to unesterified fatty acids, which is known to leave the blood extremely rapidly, would be an obvious example of this last mechanism.

Removal of Chylomicron Lipid as Particles: There is good evidence that chylomicra enter the tissue fluid and lymph directly from the blood. Thus, chylomicra have been found in the lymph from the hepatic, cervical and leg lymphatics in animals absorbing a fatty meal. The concentration of lipid in the hepatic lymph is much greater than that in other lymphatics.[45,46] Again, after the intravenous injection in the rat of chylomicra in which the triglyceride moiety has been labelled, radioactivity appears in the lymph of the thoracic duct within thirty minutes and, after two hours the level of activity is the same as that in the blood at that time. This radioactivity in the lymph of the thoracic duct is present mainly in the chylomicron fraction.[14]

Such experiments show that leakage of intact chylomicra out of the blood does occur but it is more difficult to assess the quantitative significance of the process. In the rat only about 1 per cent of the intravenously injected chylomicron triglyceride appears in the thoracic duct. However, the concentration of chylomicra in the lymph reflects the number which have not been taken up by the cells from the tissue fluid; the number actually escaping from the blood and being taken up by the cells may be much greater.

Further evidence for the removal from the circulation of intact chylomicra has been obtained by Morris and French.[47] They found that uptake and subsequent oxidation of chylomicron triglyceride occurred in the perfused liver under conditions in which prior lipolytic breakdown of the chylomicron triglyceride by the heparin clearing reaction was improbable. Approximately 8 mg. of triglyceride was taken up per hour and fat droplets were observed mainly in the parenchymal cells of the liver.

The mechanisms whereby intact chylomicra cross the capillary wall and are taken up by the tissue cells is unknown. However, in the liver, where a considerable proportion of chylomicron triglyceride injected into the blood appears,[48,49] electron microscope studies have suggested that the hepatic sinusoids may not have a continuous endothelial lining but a fenestrated structure so that the parenchymal liver cells come into direct contact with the circulating blood.[50–52] In this organ, therefore, uptake might not be governed by capillary permeability.

In tissues and organs other than the liver, electron microscope studies on the structure of the capillaries have so far not shown any discontinuities in the endothelial cell layer to suggest that direct filtration of intact chylomicra might take place.[53] Unfortunately, to my knowledge, there are no published studies with the electron microscope on the detailed structure of the capillaries in adipose tissue.

There is some evidence that the reticuloendothelial system has a role in the removal of fat from the blood. When artificially stabi-

lized lipid emulsions are introduced into the blood they are taken up by the reticuloendothelial system and particularly by the Kupffer cells of the liver.[54] However, chyle triglyceride, when injected intravenously or when taken up by the perfused liver, appears primarily in the parenchymal cells of the liver rather than in the Kupffer cells[47,55] and although cholesterol, fed to rats, appears in the Kupffer cells[56] this does not necessarily imply a direct uptake of intact chylomicra by these cells.

The removal of chylomicra from the blood, nevertheless, does show some resemblance to a phagocytic process.[14] It occurs at an exponential rate which is inversely proportional to the initial concentration in the blood. These kinetics are similar to those observed with particles known to be removed by the reticuloendotheilal system so that it is possible that the uptake of chylomicra occurs by a process resembling phagocytosis, although not necessarily by cells forming part of the reticuloendothelial system.

At present, while the liver appears to be the most likely site for the direct removal from the circulation of chylomicra as such, the quantitative significance of this process is unknown.

Degradation of Chylomicra in the Blood by the Clearing Factor Lipase: It has already been suggested that if the chylomicron triglyceride was hydrolysed to unesterified fatty acid before its removal from the blood, then this process would provide an ideal mechanism for the distribution of dietary lipid to the tissues, since unesterified fatty acids are known to leave the circulation extremely rapidly.

What evidence is there for the occurrence of such a lipolysis of the chylomicron triglyceride? First, low density lipoproteins, which could represent the products of the lipolytic breakdown of the chylomicra, appear in the blood during the absorption of a fatty meal in man.[54] Furthermore, when chylomicra in which the triglyceride has been labelled with C^{14} are injected intravenously into dogs, the specific activity of the unesterified fatty acid fraction rises progressively during the disappearance of the chylomicra from the blood.[15,58,59] Unesterified fatty acid specific activities, equal to 10 and 50 per cent of the specific activity of the injected chylomicron fatty acids, have been reported; presumably the value observed will depend on the concentration and rate of turnover of endogenous unesterified fatty acids in the blood and may vary with the nutritional conditions. The specific activity of the monoglyceride and diglyceride fractions in plasma also rises during the ingestion of labelled triglyceride.[60]

These studies indicate that lipolysis is involved in the removal of chylomicron fatty acids from the blood. Some evidence that the clearing factor lipase is specifically responsible for this lipolysis has been obtained. Thus, during fat absorption in the rat, plasma samples will clear chyle spontaneously *in vitro* by a lipolytic reaction analogous to that induced by the injection of heparin.[20,61,62] Under the optimal conditions for the demonstration of this lipolytic reaction it has been calculated that up to 30 per cent of the chylomicron triglyceride could be hydrolysed to unesterified fatty acids in the blood.

The ability of a number of substances, such as protamine sulphate and triton which inhibit the clearing factor lipase *in vitro*, to cause a lipaemia or delay the removal of ingested or injected fat from the blood in normal animals[12,13,62–64] is also consistent with an action of the lipase in the removal of dietary fat from the blood. These substances are either surface-active or can cause clumping of the chylomicra however and these factors could influence the direct passage of chylomicra through the capillary walls as well as their susceptibility to lipase action.

When chylomicra, in which either the phosphatide or protein moieties, as well as the triglyceride, have been labelled, are injected intravenously, the labelled protein and phosphatide remain in the blood for much longer than the labelled chylomicron triglyceride.[65–67] This was at first thought to provide further evidence for the degradation of the chylomicron before its removal from the blood. However, it has been pointed out that exchange of the chylomicron phosphatide and protein moieties with phosphatide and protein in the plasma lipoproteins may occur in these studies and it is difficult, therefore, to assess their significance.

Although the level of clearing factor lipase in the plasma of rats during fat absorption is considerable, it is nevertheless much below that found after the injection of heparin. Moreover, in other species, man for example, the level in the blood during fat absorption is much lower than in the rat.[68,69] This has led to the concept that the main site of action of the clearing factor lipase may not be normally in the circulating blood.

If the action of the lipase was in the tissues, on chylomicron triglyceride which had left the blood as such, then the appearance of hydrolysis products such as the unesterified fatty acids in the blood during fat absorption might be explained since these could presumably re-enter the plasma. However, the problem posed by the rapid rate of removal of intact chylomicra from the blood, at sites other than the liver, would remain. Furthermore, it is not easy to see how, after the injection of heparin, the enzyme could be released rapidly into the blood from a binding site in the tissue cells.

An alternative hypothesis to both the intravascular and extravascular sites of action of the clearing factor lipase is that the enzyme is, as has already been suggested, closely associated with the endothelial cells lining the blood vessels and that it normally acts at this site to hydrolyse chylomicron triglyceride with the liberation of the products of lipolysis (the unesterified fatty acids) into the blood. These would be distributed throughout the body fluids, according to our knowledge of their rapid transport across the capillary walls, and would be available for utilization or storage. The extremely rapid appearance of the enzyme in the circulation following the injection of heparin would be explained and a limited amount of enzyme might be released into the blood after a fat meal by absorption at the surface of circulating chylomicra.[20]

Such a site of action is plausible and would explain the rapid distribution of chylomicron triglyceride to the tissues in the absence of a high level of lipolytic activity in the blood. It is evident, however, that direct evidence in support of the idea is extremely difficult to obtain and that, at present, we cannot properly assess the relative importance of removal of intact chylomicra from the blood and the breakdown of chylomicron triglyceride to unesterified fatty acid before its removal from the blood.

The changes in the distribution of dietary fat in the tissues according to the nutritional state may eventually be explained in terms of the control of these two mechanisms of removal. It has been shown that in animals fed carbohydrate, the distribution of injected chyle in the tissues differs from that in starved animals in that there is increased uptake of chyle by the adipose tissue and decreased uptake by the liver and also, as might be expected, the amount of the injected chylomicron lipid which is oxidized directly is less.[49,70] This last effect of carbohydrate is not a specific one since the feeding of fat has an equally sparing effect on the oxidation of injected triglyceride.[71]

The simultaneous feeding of carbohydrate and fat has been reported to result in a lower degree of alimentary lipaemia than that which follows the feeding of fat alone, an effect which, it is claimed, is not due to any interference with fat absorption from the intestine.[72–74] The finding is as yet unexplained since the rate of removal of injected triglyceride from the blood is not affected by the ingestion of carbohydrate.[75]

This then can only be an interim report on the role of the clearing factor lipase in the disposal of dietary fat. I am well aware of many notable omissions. In particular, the evidence during fat absorption for a stepwise degradation of the low density lipoproteins in the blood by triglyceride removal[76] and the role of the high density lipoproteins in the structure of the chylomicrons and very low density lipoproteins have not been discussed in any detail.[67]

Mobilization of Depot Lipid

Although the possible role of the clearing factor lipase in the mobilization of depot triglyceride will be discussed elsewhere I must mention it briefly because of its interest. If an animal has adequate supplies of carbo-

hydrate and protein, both exogenous and endogenous fat is laid down in the adipose tissue for subsequent utilization as an energy source. In starvation, and in certain disease states, for example in diabetes, when carbohydrate oxidation is minimal, these fats are mobilized from the adipose tissue cells and oxidized to provide energy for the needs of the body.

Until a few years ago it was thought that the phosphatides and cholesterol esters in the plasma lipoproteins were of prime importance for the transport, in combined form, of such mobilized fatty acids from the depots. However, it is now evident that the importance of these fractions as carriers of large amounts of mobilized lipid is small since the rate of turnover in the blood of the cholesterol ester and phosphatide fatty acids is too slow to allow quantitatively significant amounts to be transported in this way. The possibility remains that these lipids are important in the transport of the essential fatty acids in the blood.

In recent years it has become increasingly clear that transport of fatty acids in the blood, in unesterified form, may be the main route for the distribution of fat from tissue to tissue and organ to organ. Dole[77] and Gordon and Cherkes[78] first reported changes in the level of the unesterified fatty acids in the blood according to the nutritional state. They found that in the fasting animal the level of plasma unesterified fatty acid rose and that the administration of carbohydrate caused the level to return to normal. They suggested that, in fasting, unesterified fatty acids left the adipose tissue via the plasma in association with the plasma albumin and were taken up and utilized by the various organs and tissues. Gordon[79] was able to measure such an uptake of unesterified fatty acids from blood perfusing the myocardium during fasting; he concluded that the amount removed was sufficient for the caloric needs of the heart muscle. At the same time as the uptake of unesterified fatty acids by the heart be found that unesterified fatty acids were released into the plasma by areas rich in adipose tissue. Both the uptake by the myocardium and the output by adipose tissue were abolished by the ingestion of carbohydrate.

The plasma turnover of unesterified fatty acids in man is sufficient to provide up to 5,000 calories per day. However, all the unesterified fatty acids turned over do not appear to be oxidized immediately in the tissues. From an analysis of data on the rate of turnover of injected labelled unesterified fatty acids and a consideration of the relation of this turnover rate to the rate of production of labelled carbon dioxide, it appears that during fasting at least 50 per cent of the caloric needs are satisfied by the oxidation of the unesterified fatty acid which has passed through the plasma.[59,80,81]

Changes in the unesterified fatty acid level in the blood in several other conditions besides starvation have also been reported[77,78,81–84] and it appears that, in general, a decrease in the utilization of glucose by the peripheral tissues is associated with an increase in the plasma unesterified fatty acid concentration. In subjects with uncontrolled diabetes, the level of the unesterified fatty acids in the plasma is high but is lowered by the administration of insulin, presumably because glucose utilization is stimulated. It has been concluded that this effect of insulin is due to a reduced inflow of unesterified fatty acid from the adipose tissue, rather than to an accelerated rate of uptake of unesterified fatty acid by the peripheral tissues.[85] This view is supported by the observation that the production of unesterified fatty acids, by adipose tissue incubated *in vitro*, is reduced in the presence of glucose and insulin.[86]

This evidence, which suggests that the level of glucose utilization controls the degree of mobilization of depot fat as unesterified fatty acid, does not rule out the further possibility that there is a direct control of mobilization by some hormonal mechanism or through the sympathetic nervous system. Epinephrine and norepinephrine, for example, have been shown to increase the level of the unesterified fatty acids in the plasma and increase the release of unesterified fatty acid from adipose tissue *in vitro*.[77,78,86,87]

In addition to the studies suggesting an

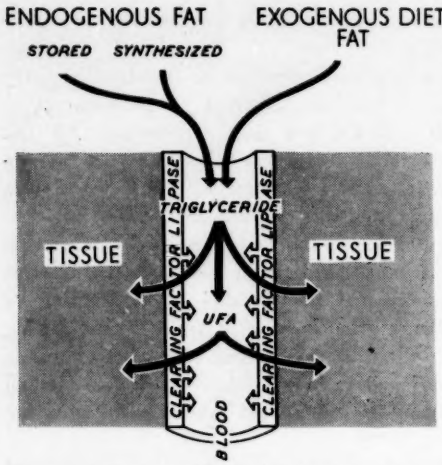

FIG. 4. A tentative scheme for the action of the clearing factor lipase.

important function for the unesterified fatty acid of the plasma in the mobilization of depot fat there is evidence that the plasma triglycerides also play a role. In starvation and in diabetes, elevations of plasma triglyceride and of the low density lipoproteins rich in triglyceride, and a lowering of these elevated levels after the administration of either carbohydrate or insulin, have been observed.[88] Moreover, lipid mobilizing fractions from the posterior and anterior pituitary have been studied and these have been shown to cause marked elevations of the plasma triglycerides.[89–92]

The turnover of triglyceride fatty acids has been found to be much greater than that of the phosphatide and cholesterol ester fatty acids.[93–95] This turnover has been reported in the dog to be 1.5 g. per hour.[96]

Since the depot fat is stored as triglyceride and this triglyceride must be broken down to unesterified fatty acids before oxidation, it is evident that lipolysis must be involved at some stage in the mobilization process. However, it is not clear at present where this lipolysis takes place.

Lipases occur in adipose tissue and the clearing factor lipase, in particular, is present in extracts of the tissue,[28,38] so that there could be lipolysis within the tissue itself. This would be in accordance with Gordon's observation during starvation that the level of unesterified fatty acid in the blood draining areas rich in adipose tissue is elevated. However, the rate of release of unesterified fatty acids *in vitro* from intact adipose tissue from starved rats is too low to account for the transport in this form of more than a small percentage of the fatty acids which would be mobilized from the depots *in vivo*.[86] Further, there is no evidence, at present, that the clearing factor lipase in the tissue is specifically involved in the release of unesterified fatty acids during starvation. The rise in plasma triglyceride levels during fat mobilization is difficult to explain if lipolysis occurs within the depot cells, although it is possible that unesterified fatty acids mobilized in excess might be reconverted to triglyceride, perhaps in the liver, and then carried back to the depots for restorage.

An alternative possibility is that the mobilization of fat from the depot cells is as triglyceride and that the unesterified fatty acids are produced from this by the clearing factor lipase acting at the capillary wall surface. Such an idea is represented in Figure 4. The observed rise in both the unesterified fatty acids and triglyceride fractions in the plasma during fat mobilization would be expected and the clearing factor lipase would play a role in the removal of mobilized fat from the blood analogous to that suggested for its action in the disposal of dietary fat. The observation that the injection of protamine sulphate during fasting causes a rise in the plasma triglyceride level[13] could also be explained by the inhibitory effect of protamine sulphate on the clearing factor lipase and, therefore, on the production of unesterified fatty acids from mobilized triglyceride.

SUMMARY

Clearing factor lipase is rapidly released into the circulating blood following the intravenous injection of small amounts of heparin. Its site of action in the body in the absence of exogenous heparin is unknown, although the available evidence suggests that it may be at the walls of the blood vessels.

Lipases must be involved in the utilization

of both dietary and depot fat since triglyceride fatty acids cannot be oxidized directly in the tissues. The evidence which suggests that the clearing factor lipase may have a specific function in facilitating transport of dietary fat to the tissues has been reviewed here. Stored triglyceride may be hydrolysed within the cells of the fat depots prior to the mobilization of depot fat. However, there is at present no evidence that the clearing factor lipase is responsible for such hydrolysis. An alternative possibility, that lipolysis occurs subsequent to the mobilization of the depot triglyceride, might, however, involve the action of this enzyme. Mobilized triglyceride would presumably be carried in the blood in low density lipoprotein complexes, and triglyceride in this form has been shown to be an adequate substrate for the clearing factor lipase.

The suggestion that the clearing factor lipase may have a general function in facilitating the transport of triglyceride fatty acids out of the blood is admittedly a speculative one. Whether or not it is supported by further work, however, the interesting properties of the clearing factor lipase, together with its general interest in relation to the problem of fat transport, fully justify our continued interest in it.

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Heparin Lipemia Clearing Reaction and Fat Transport in Man

Summary of Available Knowledge

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A BRIEF description of the state of circulating lipids is obligatory in any discussion of mechanisms involved in fat transport. Lipids do not exist in plasma as isolated molecules of free or esterified cholesterol, phospholipid or neutral fat, but are bound together with protein in the form of complex lipoproteins. These lipoproteins form a spectrum of compounds of varying size and density, ranging from the largest particles of lowest density, the chylomicrons, down to the smallest particle of highest density.[1] They can be separated grossly into α and β-lipoproteins by electrophoretic or chemical technics, and defined quantitatively by ultracentrifugal analysis into high and low density lipoproteins of varying S_f classes. In general, chemical analysis of isolated fractions has shown that as one proceeds from the largest to the smallest lipoproteins, the percentage of total and esterified cholesterol, phospholipid and protein is increased and the triglyceride content decreased.[2] Following a meal containing fats, the blood is particularly rich in chylomicrons which predominantly contain triglyceride. The triglyceride functions as the major vehicle for the transport of esterified fatty acids in plasma with the most rapid turnover rate.[3] Despite their low level in the blood, the significance of the plasma unesterified fatty acids as an important energy source has been appreciated only recently and the evidence summarized.[4]

HEPARIN

The accumulated findings of many workers in regard to heparin and the formation of a lipemia clearing factor have already been presented in this symposium.[5] Briefly, it was found in 1943 that heparin abolished alimentary lipemia *in vivo*.[6] It was demonstrated that postheparin plasma cleared lipemia *in vitro*.[7] The nature of the lipid alterations involved in "clearing" was understood when it was observed that chylomicrons and the larger low density lipoproteins rapidly disappeared from the plasma with a concomitant loss of fat from the bloodstream following the injection of heparin.[8] Subsequent investigation disclosed that a true lipolysis of neutral fat occurred with a splitting off of unesterified fatty acids and glycerol and the appearance of smaller, more dense lipoproteins.[9,10]

The essential function of albumin in this reaction as a carrier molecule for free fatty acid was then established.[11,12] Finally, the active lipolytic substance was found in the hearts of normal rat[13] and in the plasma of some human subjects without prior injection of heparin,[14] the lipoprotein nature of its substrate was defined,[15] and it was more accurately named heparin-activated lipoprotein lipase. The active lipolytic enzyme, which appears in large amounts in plasma following the injection of heparin in man, has essentially the same action and properties as in experimental animals. This paper will summarize the available evidence relative to the important question:

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"does the heparin lipemia clearing factor have a major physiologic function in the removal of postalimentary lipemia in man?" This involves a discussion of all significant studies to date dealing with human endogenous lipemia clearing mechanisms and aberrations thereof.

In 1953, using optical density technics, trace amounts of endogenous lipemia clearing activity were noted in a euglobulin precipitate of pooled human serums.[15] However, the use of distilled water, hydrochloric acid and changes in the pH raise serious doubts as to the mechanism of the slight clearing observed, since physical factors can affect optical density. Furthermore, lipases in serum may have escaped from white blood cells trapped in the contracting clot. In another report of clearing activity, which appeared in abstract form,[16] the use of small amounts of serum (which usually has much less endogenous heparin-like clearing than plasma), the absence of activity on olive oil but not on mineral oil, and the presence of greater activity in serums from subjects with atherosclerotic as opposed to serums from normal subjects indicate that the optical density decreases noted were not enzymatic and were probably due to instabilities in the clearing system used. The presence of endogenous lipemia clearing (twenty-five cases) and lipolytic activity (one case) in the plasma of some normal subjects was then definitely shown.[14,17] The inhibition characteristics of the endogenous clearing factor were similar to those of the postheparin enzyme, and there was no correlation with serum tributyrinase. Subsequently lipolytic activity was demonstrated *in vitro* in the preheparin plasma of nine subjects. In each of the subjects identity of the endogenous lipolytic factor with heparin-activated lipoprotein lipase and its distinction from pancreatic lipase was established by inhibitor studies.[18]

Other investigators had also noted endogenous lipolytic activity in a human plasma sample after a meal containing fat, but the nature of the enzyme involved was not identified.[19] The occasional presence of minor degrees of circulating lipolytic or clearing activity has since received further confirmation[20–24] by *in vitro* studies. The finding of increased amounts of plasma unesterified fatty acids *in vivo* following a fatty meal[19,25] had not proved that triglyceride lipolysis subsequent to intestinal absorption had occurred, since these acids may be found in the lymph[26] after the intake of food. Additional evidence of the identity of human endogenous plasma clearing factor with postheparin lipoprotein lipase and its distinction from pancreatic lipase, has been afforded by studies using tricalcium phosphate gel which adsorbs heparin and postheparin clearing activity,[27,28] but not human pancreatic lipase.[29] The endogenous plasma lipolytic activity is completely or almost completely removed by the gel.[29]

At this point it may be well to discuss briefly the presence of heparin in normal blood, since doubt on this score has even been expressed in recent publications. It is true that assay methods which are based on the neutralizing action of heparin-combining agents in the clotting or clearing reactions are non-specific. Furthermore, extraction methods in which the end-product is assessed by its metachromatic activity do not afford proof of the existence of heparin. Two groups of investigators working independently with different extraction methods have isolated a substance from normal human plasma which has the biologic properties of heparin, namely, anticoagulant and antithrombic activity.[27,30,31] The plasma extract was neutralized by protamine and moved identically with commercial heparin when subjected to electrophoresis on toluidine blue paper.[31] Although normal limits have not been precisely delineated, it would appear that approximately 10 to 20 units per 100 ml. (1 mg. = 100 units) is the usual range of circulating heparin in man. This totals 3 to 6 mg. in a plasma volume of 3 L., a considerable quantity of heparin and certainly sufficient to function physiologically.

LIPOPROTEIN LIPASE

The formation of postheparin lipoprotein lipase has been demonstrated in all organs except the liver and the brain in animals,[32–35] but this has not been determined in man. Inactivation of lipoprotein lipase by the liver in animals[35,36] and in man,[37] has been demonstrated. Thus far in man the enzyme has been

found only in the bloodstream. There is no published evidence of its presence in human tissue. Various reports have mentioned inhibitors of postheparin clearing factor in serum from normal subjects,[38-40] in serum from patients with atherosclerosis[41] and in serum from patients with essential hyperlipemia.[42,43] Only optical density changes were followed in each of these studies and thus inhibition of lipolysis remains unverified. Optical density changes are deceptive, and it has been clearly shown that triglyceride lipolysis by postheparin serum may be proceeding in a normal fashion in the presence of an inhibitor of clearing.[42,44] No differences were found in the inhibitory property between normal subjects and those with atherosclerosis to explain the differences in lipids in the two groups. The inhibitor did not interfere with the clearing factor response to small doses (3 mg.) of heparin.[39] Therefore, it seems reasonable to conclude that circulating inhibitors of lipoprotein lipase are not present to any important degree if they are present at all. However, thrombin,[45] extracts of white blood cells and platelets[46] and human tissue extracts[47] inhibit lipolysis to some extent. The mechanism, specificity or possible role of these substances is unknown.

The endogenous enzyme is present more frequently and has greater activity in plasma than in serum aliquots.[17,48] This difference is apparently due to its adsorption by fibrin since most or all of the clearing activity is lost when active plasma is defibrinated by shaking with glass beads.[49] This distinction between plasma and serum levels is not true of tributyrinase or pancreatic lipase; it frequently cannot be demonstrated using postheparin blood, as much larger amounts of the enzyme are usually present under these circumstances.

Endogenous human lipoprotein lipase is lipolytic when postlipemic chylomicrons or low density lipoproteins are the substrate, but whether or not it has any activity upon neutral fat emulsions has not been investigated to my knowledge. Attachment of the postheparin active factor to chylomicrons has been shown,[50,51] but similar studies of the endogenous human enzyme have not been made. Presumably, in this case, linkage of substrate and enzyme also occurs before lipolysis. This bond probably consists of a linking between the protein or phospholipid of the lipoprotein and heparin. The presence of heparin as part of the enzyme is indicated by its inactivation with protamine,[17,18] but recently much stronger evidence has been presented.[52] In several instances it was found that the simple *in vitro* re-addition of heparin to the inactive supernatant of both postheparin and endogenously active plasma after tricalcium phosphate gel adsorption (which removes heparin) partially restored the lipemia clearing activity. This finding, although it remains to be confirmed by studies of lipolysis, indicates that heparin is a component of lipoprotein lipase, perhaps as a prosthetic group. The inactivation of the lipoprotein lipase of chicken adipose tissue by bacterial heparinase[53] suggests that this is also true in animals. However, this does not constitute complete proof, as heparinase also inactivates other sulphated mucopolysaccharides.[54]

Heparin apparently stabilizes the enzyme activity,[55] mobilizes it and effects its rapid attachment to the lipoprotein substrate. The occasional reconstitution of activity of the supernatant plasma by the addition of heparin after tricalcium phosphate adsorption shows that in some cases the apoenzyme of lipoprotein lipase was not as completely adsorbed by the gel as the heparin, and partially remained in the plasma. This suggests that the bond between heparin and apoenzyme is easily broken. The experiment further indicates that the formation of lipoprotein lipase does not require tissue function or energy, consisting simply of a union of heparin and apoenzyme. The latter is the true lipolytic moiety. Apoenzyme apparently is normally present in tissue and is released by, and loosely bound to, heparin.

INACTIVATION

These matters have been discussed in some detail as they are also pertinent to an unusual feature of human lipoprotein lipase; namely its inactivation during lipolysis. This inactivation was first suggested when studies of plasma lipolytic activity before and after the oral intake of fat were reported.[56] Additional cir-

cumstantial evidence was obtained following the intravenous administration of fat emulsions.[37] Recent *in vitro* studies using postheparin and endogenously active plasma have proved that loss of enzymatic activity occurred as lipolysis proceeded in the presence of excess substrate.[52] This was not true of human pancreatic lipase, suggesting, in view of the findings outlined in the preceding paragraph, that lipoprotein lipase activity proceeds in the following manner:

The heparin component of the enzyme combines with the protein or phospholipid moiety of the chylomicron or lipoprotein molecule. This brings about contact between the apoenzyme and its substrate, the triglyceride of the lipoprotein, and lipolysis ensues. At the same time, the union of heparin and apoenzyme is broken and further lipoprotein lipase activity is absent or unstable. This concept is speculative, but seems to fit the observations made thus far. The accuracy or inaccuracy of this proposed mechanism does not affect the significance of the demonstration of enzyme inactivation during lipolysis. The implications will be discussed later.

EFFECT OF FAT

The stimulating effect of intake of olive oil upon plasma lipemia clearing factor was first observed in rats.[57] It was of short duration and disappeared within a half hour after the fat stimulus was abruptly stopped by ligation of the thoracic duct.[58] In man the increased electrophoretic mobility of the α- and β- serum lipoproteins seen, after the intravenous administration of fat emulsions resembled that observed after the injection of heparin.[59] More direct evidence of the stimulation of endogenous plasma lipolytic and clearing activity after oral and intravenous intake of fats was then presented; the enzyme involved was identified by inhibitor studies as lipoprotein lipase.[37,56] In parallel experiments using two different methods of heparin extraction and assay, an average increase of 22 per cent in circulating heparin levels was found in twelve of eighteen human subjects during or directly after the intravenous administration of a fat emulsion, whereas a lesser rise occurred in four of fourteen patients after the administration of an identical control non-fat emulsion.[60] These results were probably significant statistically. Similar findings were obtained after the oral intake of fat[61] but statistical significance was not established as a more rapid entrance of fat into the blood and a higher level of lipemia occurred after the intravenous administration of the emulsion. Lysis of mast cell granules and diffusion of metachromatic material (presumably heparin) into the surrounding tissue has also been observed in rats after the intravenous injection of an emulsion containing olive oil; whereas injections of the emulsion without oil gave no such results.[62]

The accumulated evidence thus indicates that fat intake is a stimulus for the release of heparin and lipoprotein lipase into the bloodstream in man. Attempts to quantitate the *in vivo* splitting of neutral fat by this lipolytic mechanism have been hampered by the dynamic state of fat transport. Following a meal, fats are continually entering the circulation via the thoracic duct and are also being removed perhaps by several pathways. *In vitro* experiments avoid some of these difficulties but suffer from the fact that the end-products of lipolysis, the unesterified fatty acids and glycerol, accumulate and may inhibit the reaction.[63] Therefore, it seems probable that even when it can be demonstrated in blood, the lipolytic process proceeds more rapidly *in vivo* than it does in the test tube. Nevertheless, *in vitro* studies of the lipolysis of postalimentary lipemia have demonstrated triglyceride lipolysis due to endogenous plasma lipoprotein lipase, at a rate varying from 0 to 5.4 mEq./L./hour[29,54,64] [in one subject a rate of 6 mEq./L./hour was found[49]]. The higher rates are sufficient to account for the splitting of 4 to 5 g. of neutral fat per hour, equivalent to the fat content of an average meal. The smaller quantity of circulating lipoprotein lipase activity reported by other investigators[21–23] results from the use of serum rather than plasma in their studies, and the measurement of total release of fatty acid or glycerol after a prolonged interval rather than calculations of enzymatic activity based upon initial rates of lipolysis. In view of the inac-

tivation of the enzyme during lipolysis, its stimulation by fat intake, and its destruction by the liver, the amount of enzymatic activity determined *in vitro* in any particular blood sample may bear little or no relationship to the immediately preceding or subsequent *in vivo* events. An enzyme, predominantly released after fat intake and inactive after substrate attachment and triglyceride lipolysis has occurred, is only accidentally demonstrated. Therefore, our inability to find substantial quantities of lipoprotein lipase activity in the plasma of the majority of subjects at any one time does not constitute a valid argument against its functioning at that site. On the contrary, the high lipolytic rates present in the plasma of several subjects are evidence of its normal role.

Optical density changes do not exactly parallel lipolysis especially when minor quantities of lipoprotein lipase are involved. Nevertheless, a recent report of studies of endogenous plasma lipemia clearing activity in 482 subjects has clarified some aspects of this subject.[65] In these experiments coconut oil, containing fatty acids of chain length C8-C18, was used as a substrate so that simple esterases did not contribute to the results observed. Eighty-nine subjects were tested on several occasions, and clearing activity was found in 112 of the 482 patients (23 per cent). An 18 per cent incidence of positivity would have been obtained if only one test had been performed in each case. It may be that a slightly lower proportion of the general population contains demonstrable plasma lipemia clearing factor at any one time, since the initial twenty-five subjects tested were known to have low lipid levels. When clearing activity was found in the first examination, it was more apt to be present on repeated testing. When it was initially absent, it was more likely to remain so on further tests. There were no children under ten years of age in the group. In subjects aged from ten to seventy-nine years there was no age or sex difference in the incidence of clearing factor. It may be significant that the latter was present in six of the eight subjects over eighty years of age.

In seventy-six of the 112 people with endogenous plasma clearing activity the effect of inhibitors or of tricalcium phosphate adsorption was evaluated. The calcium phosphate adsorption was used most often. In sixty-nine of the seventy-six subjects these procedures inhibited or removed the clearing factor, thus indicating its identity with lipoprotein lipase in 90 per cent. In three of the seven remaining subjects studies of unesterified fatty acid release upon incubation of lipemic plasma were carried out; no lipolysis was demonstrated although clearing was present. Thus, in about 10% of the cases in which it is present, endogenous clearing activity is non-lipolytic, a physical phenomenon rather than a splitting of triglyceride. In thirty-two subjects, with and without clearing factor, glycerol release was measured along with optical density changes. It was found that minor degrees of lipolysis may be present in the absence of clearing. However, when decreases in optical density occurred, glycerol release was increased, establishing a poor correlation between endogenous clearing and lipolytic activity.

Cholesterol was determined in 364 of the 482 subjects; the low density lipoproteins were analyzed in 234. There was no difference in the average cholesterol values between those who had clearing activity and those who did not. However, both the S_f 0–12 and the S_f 12–400 lipoproteins were higher in the latter group. This difference in the S_f 12–400 lipoproteins (high in neutral fat content) was statistically significant, $p = 0.01$, almost significant in the S_f 0–12 class. These results are in agreement with the previously published findings of an inverse relationship between plasma heparin levels and circulating low density lipoproteins.[66] They add considerable weight to the existing evidence which indicates the physiologic relationship of heparin and lipoprotein lipase to the removal of ingested fat from the bloodstream.

Surprisingly, clearing activity was not found more frequently in premenopausal females than in males of the same age, although levels of low density lipoproteins are considerably lower in the former. Clarification of this point must await further investigation. Although the average low density lipoprotein levels were

lower in patients in whom clearing factor was found, many others had low lipid values without clearing activity. This is not unexpected in view of the previously discussed stimulation of lipoprotein lipase upon fat intake and its inactivation as triglyceride lipolysis occurs. Nine patients with high lipid values had a positive plasma. In four of the nine results of liver function test were abnormal thus demonstrating that in this situation the correlation between lipemia clearing factor and lipoproteins is affected by factors other than enzyme-substrate relationships. In the remaining five patients there was no obvious explanation of the presence of circulating lipoprotein lipase together with elevated levels of low-density lipoproteins.

On several occasions, in the presence of both endogenous and postheparin lipoprotein lipase, decreases in glycerol and free fatty acid concentration were observed *in vitro* after an increase had occurred.[48,65] This indicates that the enzyme may also function in the resynthesis of triglyceride, an action known to occur with pancreatic lipase. A similar suggestion has been made based upon studies using human postheparin factor and rat chylomicrons.[67] It has also been found that the postheparin enzyme attacks the triglyceride component of chylomicrons in a manner quite similar to the action of pancreatic lipase.[67]

Investigations of clearing factor following the injection of heparin in man may contribute valuable information about the availability of the tissue factor necessary for the formation of lipoprotein lipase. Unfortunately the results of various studies are in conflict. Several investigators have stated that the development of clearing activity after the administration of small doses of heparin is less in patients with atherosclerosis than in normal subjects,[68–73] and is lower in older age groups.[71,74,75] Others have not found any significant difference in clearing activity after the administration of small or large doses of heparin to normal subjects or to those with atherosclerosis,[39,74,76,77] nor was any variation in response to small doses noted with age, using a rigidly standardized technic.[77] A general criticism of all these studies, particularly those in which small doses of heparin were administered to patients with atherosclerosis, is that the plasma neutral fat was not measured. This lipid substrate may have been available in varying amounts in the plasma, therefore lesser degrees of lipoprotein lipase activity would have functioned and been inactivated during lipolysis before the postheparin sample was withdrawn for *in vitro* assay. Using larger doses of heparin, injected by various routes, ample triglyceride lipolysis was demonstrated *in vitro* in eight patients with atherosclerosis, although there were individual variations.[78]

In the majority of cases tissue factor deficiency does not appear to be a barrier to the therapeutic use of heparin, or a limiting factor physiologically, in view of the statistically significant inverse correlation between circulating heparin and low density lipoproteins.[66] One might argue that if heparin is a component of lipoprotein lipase, lack of the tissue factor would result in lower levels of heparin. However, this is not reasonable. After the injection of small doses of heparin lipoprotein lipase activity is found very quickly; adequate heparin is circulating in postabsorptive blood whether or not the lipolytic activity is present, and free metachromatic material has been observed diffusing from mast cells after the injection of olive oil in rats.[62]

PHYSIOLOGIC STUDIES

Thus far the available evidence has been outlined proving the existence of an endogenous lipolytic enzyme in man which is quantitatively capable of functioning in the removal of alimentary absorbed neutral fat from the bloodstream. What role does it normally occupy in that important function? At this point many studies in animals relative to this subject must be evaluated, although species differences probably exist. These experiments have been concerned with the role of the reticuloendothelial cells functioning as a phagocytic system in the disposal of ingested and injected fat and cholesterol. One of the earliest investigations[79] clearly indicated that lipids were removed from the blood by different routes when they were administered as normal chyle or as artificial fat emulsions. In the latter instance, fat was

treated like a foreign material and rapidly appeared in the lungs, splenic pulp and the hepatic Kupffer cells, whereas this never occurred with chyle. In this study in dogs and rats no evidence was obtained of phagocytosis of chyle lipid by the reticuloendothelial system. Substantiation of the minimal role played by reticuloendothelial phagocytosis in rats was afforded by experiments[80] in which particle takeup by reticuloendothelial cells was blocked by Trypan blue and carbon black. In these circumstances injected fat emulsions were removed from the plasma at a normal rate.

Other investigators, however, found labeled ingested cholesterol in the hepatic Kupffer cells of the rat[81] which they believed indicated "intake by the cells of cholesterol as oriented particles, most probably in the form of chylomicrons." No observations were advanced in support of this viewpoint, and it is certainly equally possible that cholesterol entered these cells subsequent to chylomicron dissolution elsewhere, or following the exchange of the unesterified cholesterol of chylomicrons with that of higher density lipoproteins.[82] Observations of impaired removal of cholesterol and lipids from the blood after blocking the reticuloendothelial system in animals with India ink[83,84] do not prove that chylomicron phagocytosis normally occurs to any substantial degree, since the ink may alter the substrate lipid particles making them unavailable to enzymatic lipolysis. Furthermore it may be that reticuloendothelial cells function in the formation of heparin lipoprotein lipase. It was found in dogs that the lipemia clearing effect of heparin was reduced for several weeks after the injection of thorium dioxide.[85] Further evidence against reticuloendothelial system phagocytosis of lipid particles is provided by the finding[86] that the deprivation of insulin in depancreatized dogs slows the removal of injected emulsified fat as does the production of mild acidosis. The latter, and the absence of insulin, have no known inhibitory effect on phagocytosis. Finally it is probable that the surface properties of intact chylomicrons would interfere with their ingestion.

Another possible pathway for the removal of chylomicron from the blood is via direct passage into the liver sinusoids which can apparently occur to some degree,[80,87] particularly when there is distention of the portal sinusoids. However, transport of large quantities of chylomicrons across the capillary endothelium is most unlikely[88,89] when the size of these lipid particles and that of capillary pores is considered. The quantitative role of the liver as a primary factor in the removal of chylomicron remains to be more clearly defined, although only 0.4 per cent of chylomicron fat appeared in the liver lymph in cats.[87] Certain clinical observations, which will be discussed later, have shown that liver function is not demonstrably abnormal in most patients with hyperlipemia and hyperbetalipoproteinemia. This would argue for the dominant role of other mechanisms in fat clearing. The delayed removal of fat emulsions in hepatectomized animals[90] simply indicates the importance of the liver in fat transport processes, not that direct chylomicron uptake by liver cells is the major channel for their exit from the blood. It has been pointed out that hepatic lymph is limpid, resembling serum in composition, and therefore, if the liver picks up particulate lipids, it converts them to lipoproteins.[91]

ALIMENTARY LIPEMIA

There is now an impressive array of experimental and clinical facts which strongly suggest that triglyceride lipolysis by the heparin lipoprotein lipase system is the major pathway for the clearance of alimentary neutral fat from the blood stream. It is this enzyme system, identified in both animals and man, which most rapidly hydrolyses the triglyceride component of serum chylomicrons and low density lipoproteins. This fact alone, in view of the controlling role of enzymes in metabolic processes, is indicative of its important role in fat transport. The rapid unidirectional stepwise conversion from larger to smaller lipoprotein species which has been described in normal man after the ingestion of a fat meal[2] and following the injection of labeled low density lipoproteins,[92] is consistent with enzymatic hydrolytic triglyceride degradation in the blood. Similar results have been obtained in animals given injections of various isolated lipoprotein classes,[93] and loss of

fat from the bloodstream occurred together with the lipoprotein changes. However, these various observations *in vivo* did not prove that lipolysis occurred nor did they identify the enzymatic mechanisms involved. The rise in unesterified fatty acids in human plasma observed after the intravenous administration of fat emulsions,[94] although strongly suggestive of lipolysis, did not establish it, as the free fatty acid content of the emulsion was not determined. Similarly the identification of mono- and diglycerides in human[95] and rat[96] plasma after fat feeding was not conclusive, as these partial glycerides may have been original constituents of the chylomicrons, or perhaps were returned to the circulation after extravascular hydrolysis. The latter possibility is most unlikely, as the specific activity of these glycerides was too high to be compatible with the dilution to be expected from a tissue process. The disappearance of labeled injected chylomicrons with a half-time of fifteen minutes, and the rapid simultaneous increase of the specific radioactivity of the plasma unesterified fatty acids, established that most of the radioactivity which disappeared from the chylomicrons appeared in the unesterified fatty acid fraction.[97] These data suggest an intimate relationship between the removal of chylomicrons from the blood and the lipolysis of their triglyceride component, although the site at which this occurred was not established. The rapid disappearance of labeled triglyceride from the bloodstream had been described previously[3,98] and was subsequently confirmed.[99,100] That disappearance of labeled chylomicro- triglyceride is more rapid than that of labeled chylomicron phospholipid from the blood[101] argues against the particulate removal of intact chylomicrons. If the latter took place to any extent, such a marked difference should not exist between these chylomicron components unless phospholipid was rapidly returned to the blood undiluted by any extravascular pool.

Indirect evidence initially implicated endogenous heparin in the normal removal of fat from the bloodstream. It was based upon the *in vivo* increase in lipemia and low density lipoproteins in animals[58, 102–105] and in man[106–108] following the injection of protamine or toluidine blue, substances which inhibit heparin. However, their mode of action may not be entirely due to the antiheparin effect. They may also act upon the surface properties of chylomicrons and so hamper their removal from the blood by pathways other than the heparin clearing system. It is known that other surface-active agents cause hyperlipemia without any direct antiheparin action.[109] Nevertheless, in this instance the lipemia is probably due to the inability of heparin lipoprotein lipase to split the altered triglyceride containing lipoproteins.[110] More direct evidence of involvement of the endogenous heparin lipemia clearing system has been forthcoming. As already mentioned, an enzyme has been identified as lipoprotein lipase in the plasma of sixty-nine human subjects,[65] and in several instances sufficient endogenous activity was found to account for the lipolysis of 4 to 5 gm. of neutral fat per hour,[64] the equivalent of a high fat intake. The statistically significant inverse correlation of this plasma enzyme,[65] and of heparin levels[66] with the low density lipoproteins adds much weight to the argument that this mechanism is the major normal pathway for the clearing of alimentary lipid from the blood. The stimulus that lipemia provides in increasing the levels of heparin in plasma and lipoprotein lipase activity should not be ignored.[37,56,60] Since this heparin-linked enzyme affects triglyceride lipolysis and the removal of fat from the bloodstream, it is probable that its increase after fat intake represents a normal response to a physiological demand.

CLINICAL ASPECTS

Clinical observations supporting this thesis have not been sufficiently emphasized. In the majority of patients with the nephrotic syndrome or with essential hyperlipemia there is no known defect in reticuloendothelial or liver function. However, an interference, of one type or another, with the triglyceride lipolytic mechanism does exist and these patients have a marked elevation of plasma neutral fats. In nephrosis there is a lack of circulating albumin, which functions[11,12] as

the carrier of unesterified fatty acids. Studies by various investigators have shown that in this disease, in man and in animals, the only demonstrable defect responsible for the increased serum lipids is lack of the acceptor protein, albumin.[92,111–114] When plasma albumin rises there is a decrease in lipid levels.[113,115]

In patients with essential hyperlipemia several types of abnormalities have been described which markedly hamper the lipoprotein lipase system. In one patient the chylomicrons were a poor substrate for the post-heparin enzyme.[116] In three siblings with the disease little lipoprotein lipase activity appeared following the injection of heparin, indicating a deficiency of the tissue apoenzyme of lipoprotein lipase.[117] In several other subjects with hyperlipemia low levels of circulating heparin were found, but clearing factor formation after the injection of heparin was excellent.[49] Thus in these cases a lack of sufficient endogenous heparin was apparently at fault.

Studies of liver function including the bromsulphalein test, in many patients with elevated levels of serum low density lipoproteins, have given no indication of liver or reticuloendothelial dysfunction.[49] Therefore in man the clinical and laboratory evidence available to date shows that the heparin lipoprotein lipase mechanism has the central role in the clearing of alimentary lipemia from the bloodstream. Other pathways for fat removal undoubtedly exist, but they are subsidiary and usually do not compensate fully unless fat intake is reduced substantially.

COMMENTS

The opinion has been repeatedly expressed that the site of action of lipoprotein lipase is not intravascular, and that chylomicrons have to be removed from the circulation intact at least as far as the cell wall, before they can be hydrolyzed. This point of view is based upon the identification of lipoprotein lipase in certain tissues, and upon the fact that the circulating enzyme either is not easily found or has minimal activity. In rats the tissue distribution of labeled palmitic acid injected as chylomicrons was quite different than when it was given as unesterified fatty acid.[118] This was interpreted as proving that the bulk of the chylomicrons did not undergo intravascular hydrolysis. However, the situations are not comparable since the splitting of chylomicrons release glycerol and probably smaller lipoproteins in addition to the fatty acids, and these may affect the tissue distribution of the latter. Furthermore chylomicron lipolysis probably would not result in as rapid a release of fatty acid into the blood as occurs following their injection. Similar objections can be raised to other recent studies in dogs in which it was concluded that only 50 per cent of an injection of labeled triglyceride fatty acid had appeared in the blood as unesterified fatty acid, and that chylomicrons as such are a plasma vehicle for the transport of fat which is directly available for oxidation.[119] In addition unproved assumptions were used in the mathematical calculations involving the radioactivity data. Finally, in all experiments in dogs it should be remembered that this species normally has a serum lipase which is presumably pancreatic in origin and its stimulation by an injection of chyle may be entirely different from that observed after the ingestion of fat.

The observations outlined earlier in this presentation have shown that plasma lipoprotein lipase is demonstrable frequently, and that occasionally high levels of lipolytic activity are present in man. The inactivation of the enzyme during triglyceride lipolysis, an unusual occurrence for an enzyme, is probably responsible for the failure to identify it in the plasma of most normal subjects after the ingestion of a fat meal. The opposing concept, namely chylomicron hydrolysis at the tissue cell wall, demands a satisfactory explanation of the exit route of these large lipid particles from the circulation. The liver is the only organ into which a moderate amount of chylomicrons may penetrate under normal circumstances. This organ normally contains esterases rather than lipase. The liver inactivates lipoprotein lipase, and this enzyme has been found there in only one study.[120] In this instance the use of minced tissue brei as the enzyme source, and the failure to use inhibitors, raise serious doubts as to the nature

of the lipase involved. To my knowledge there are no published reports of the identification of tissue lipoprotein lipase in man. Although much more investigation is necessary, thus far intravascular alimentary triglyceride lipolysis by lipoprotein lipase has been demonstrated in man, whereas the extravascular site of action by the same enzyme has not. It is, therefore, more reasonable to consider that the burden of proof in this matter lies upon those who favor extravascular lipolysis. The finding that lipoprotein lipase is released quickly after the injection of heparin has led to the suggestion that the enzyme is available in the capillary endothelium and acts at the capillary wall.[5] This is possible and, if true, still represents an intravascular phenomenon.

The available evidence in man dealing with mechanisms involved in the removal of alimentary triglyceride from the blood is reviewed. In our opinion the data support the thesis that the major normal pathway is by way of intravascular enzymatic lipolysis by heparin lipoprotein lipase.

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Lipid-Induced Coagulation Changes in Normal Subjects and in Patients with Atherosclerosis

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IN 1951 Swank[1] described the effects of a high triglyceride challenge on clumping of chylomicra and packing and distortion of red cells. In his experiments the chylomicra remained separate until about six hours after the administration of fat. At that time they would tend to aggregate, first as doublets and triplets and then as larger clumps. The red cells, which were largely dissociated in fasting blood samples, formed rouleau and at about six hours after the challenge these cells became more tightly packed and distorted. In some cases the cluster of red cells gave the appearance of an amorphous mass when viewed in dark field illumination at × 1,000 magnification. After nine to twelve hours the serum was clear of clumped chylomicra and the red cells returned to normal. The dose necessary to produce these effects was 2 gm. of fat per kg. body weight in man and double that in dogs. A lesser dose of 0.5 or 1 gm. per kg. did not give as profound a response.

Later, Cullen and Swank[2] administered a similar dose of cream to hamsters and observed the intact circulation in the pouch of the cheek. These investigators were able to observe the same clumping and adhesiveness of red cells *in vivo* and also observed that the rate of circulation was halted for long periods of time in the peripheral vessels. As in the case of the *in vitro* observations, these circulatory effects did not occur until about six hours after the challenge or after the peak of lipemia. The circulation returned to normal after about twelve hours.

As these studies demonstrated an effect of a high fat challenge on integrity of red cells and on rate of circulation in the peripheral vessels, it appeared worth while to investigate the effect of such a lipid challenge on coagulation of whole blood at the times when these circulatory changes were taking place.

Several studies of the effect of fat on coagulation employing less than 2 gm. of lipid per kg. body weight have been carried out. No significant effect on the coagulation system has been established at these dose levels. A number of studies have now been undertaken utilizing a higher lipid challenge and the results are controversial. In none of these studies, however, was the blood coagulation measured at the time demonstrated by Swank to be critical with respect to rate of circulation and packing and distortion of red cells.

Mustard,[3] Buzina,[4] Maclagan[5] and Fullerton[6] are some of the investigators who found that clotting time was shortened following a high dose of fat. Sheehy,[7] Borrero[8] and Sohar[9] were not able to demonstrate a significant shortening of clotting time following a fat meal.

METHOD

Evaluation of these studies revealed a difference in the technics employed to measure

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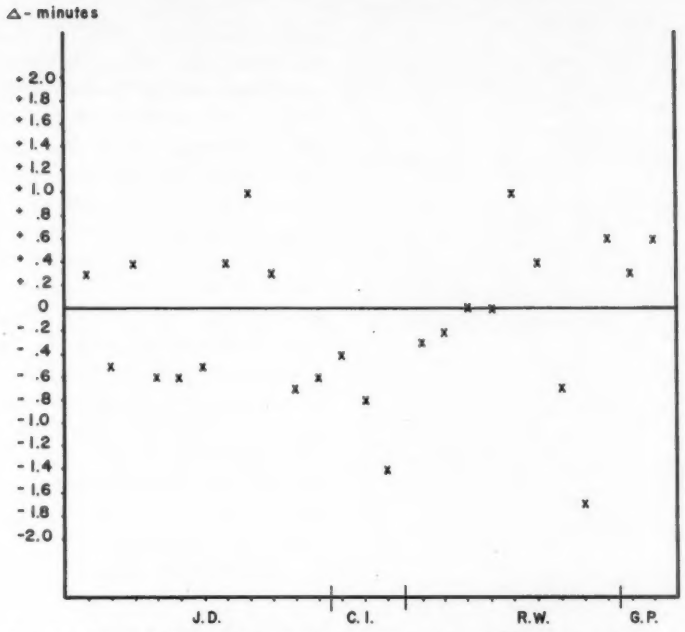

FIG. 1. Reproducibility of method: differences in coagulation time between twenty-five sets of two control samples drawn thirty minutes apart.

coagulation values and, in several of the cases, a considerably greater variation in baseline values than is desirable. In order to be in a position to evaluate small changes in coagulation, a modification of the silicone tube whole-blood clotting method was devised. The details of this method will be described elsewhere.[10] The reliability of the method was measured by comparing the fasting clotting times of two samples of blood from the same subject drawn thirty minutes apart. The difference between the clotting times of twenty-five sets of punctures are indicated in Figure 1. The variability of the method in measuring coagulation of samples at two time periods is 0.54 ± 0.15 minutes (P = 0.95 limits). The greatest difference between the coagulation times of the first and second samples in any set of punctures was 1.7 minutes. The baseline clotting time by this method varied with the pretreatment of tubes and was about thirty minutes.

EXPERIMENTAL METHODS

Ten normal men, aged twenty-eight to forty-five years, were given 2 gm. of fat as whipping cream per kg. body weight after a fasting blood sample had been drawn. Additional blood samples were obtained at two, four, six, eight and ten hours after the challenge. Chylomicra, times of packing and distortion of red cells and coagulation of whole blood were measured. No additional lipids were consumed by the subjects during the study. Coffee and tea were allowed throughout the day and crackers and shrimp were eaten after the eight-hour puncture.

The same ten subjects were again challenged with 280 ml. of skimmed milk and 1,260 calories of carbohydrate following a baseline puncture. The carbohydrate was consumed over a one-hour period and consisted of sucrose, jam, toast, bananas and apple sauce. Fasting blood samples were obtained and additional samples were obtained at two-hour intervals. Coffee and tea were permitted and a fat free meal was consumed after the eight-hour puncture.

Through the courtesy of Dr. Eugene Chesrow and with the assistance of Dr. Joseph

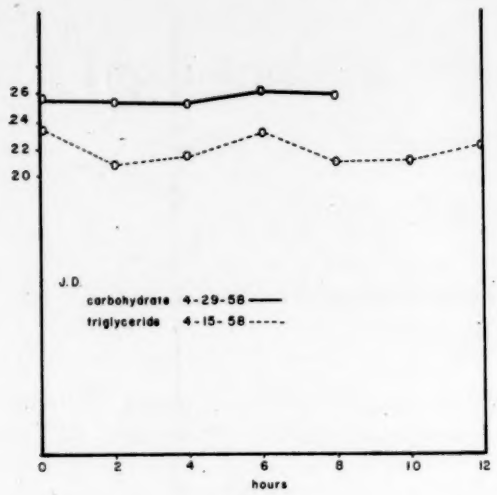

FIG. 2

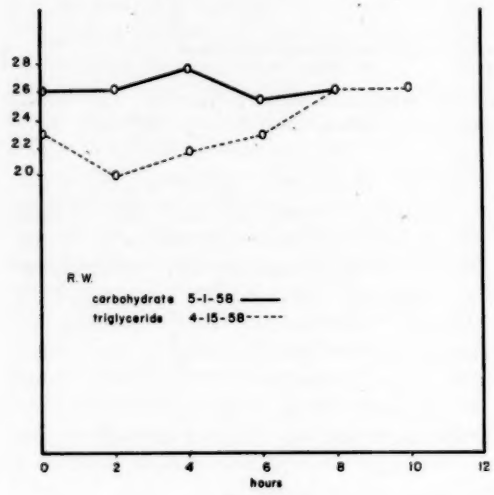

FIG. 3

FIGS. 2 and 3. Coagulation time in minutes following lipid and carbohydrate challenge.

Musci of Oak Forest Hospital, Chicago, five male and five female patients, aged forty-one to seventy-two years, who had had a cerebral vascular accident, were challenged with 2 gm. of cream per kg. of body weight in the manner described above. A fasting blood sample was obtained and additional punctures were made at approximately two-hour intervals.

RESULTS

Normal Subjects: The whole-blood coagulation time of either the two- or four-hour sample from each subject was shortened following the triglyceride challenge. By the sixth hour the coagulation time had returned to baseline in eight of the ten subjects. A shortening of coagulation time was demonstrated at either the sixth or eighth hour in eight of the ten subjects, while the coagulation time was at baseline or above in the other two subjects. The maximum drop at the two-or four-hour reading was 18.2 per cent and the maximum drop at the six- or eight-hour reading, coinciding with maximum packing and distortion of red cells, was 17 per cent. Figures 2 and 3 represent examples of the response obtained.

Similar deviations from baseline coagulation values were not obtained when the normal subjects were subjected to an isocaloric carbohydrate challenge. The average of the readings at the two- or four-hour low showed no drop, the average of the readings at the four- or six-hour high was 107 per cent of baseline and the average of the readings at the six- or eight-hour low was essentially the same as baseline.

Patients with Cerebrovascular Disease: These patients responded to the lipid challenge with coagulation times of 58.3 to 95.6 per cent of baseline at either the second- or fourth-hour puncture. The rebound values at either the fourth- or sixth-hour puncture ranged from

TABLE I
Average Coagulation Values for Each Group of Ten Subjects

Challenge and Subject	Postchallenge Coagulation Values (Per Cent of Baseline)		
	2–4 Hour Low	4–6 Hour High	6–8 Hour Low
Triglyceride, normal subjects	89.7	96.6	95.7
Carbohydrate, normal subjects	100.0	107.2	99.0
Triglyceride, patients	81.6	96.9	78.1

87 to 108 per cent of baseline. A second drop in coagulation time at either the sixth- or eighth-hour puncture was evidenced by coagulation values ranging from 62.8 to 87.6 per cent of baseline. This second depression in coagulation time coincided with maximum packing and distortion or red cells. The averages of these values compared to the normal group are indicated in Table I.

CONCLUSIONS

The whole-blood coagulation time following a lipid challenge was shortened in both normal subjects and patients with cerebrovascular disease. This effect was measured utilizing a coagulation method of low inherent variability. The coagulation time curve had two depressions, one at two to four hours and one at six to eight hours after the challenge. The shortening in coagulation time at two to four hours after the ingestion of cream confirms earlier studies. The drop in coagulation time at six to eight hours after the challenge corresponds to the time of maximum packing of red cells and decreased rate of circulation reported by Swank. This second shortening in coagulation time was greater in the older patient group than in the normal subjects. An isocaloric carbohydrate challenge to the normal group did not produce a similar alteration in coagulation time of whole blood.

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Freely Extractable Lipid of Human Blood Plasma

I. Methodology and Observations in Normal and Abnormal Subjects

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LIPID solvents in use in biologic laboratories may be divided generally into those that cause a complete breakdown in lipoprotein complexes, and those which extract only a small portion of the plasma lipids with resultant minimal disruption of the basic lipoprotein complexes. The latter group includes pentane, hexane, benzene, chloroform and a number of others.

Forbes[1] extracted some cholesterol from lyophilized plasma with cold chloroform and called this fraction "unbound cholesterol." Byers,[2] using a different solvent system (10 per cent ethanol and 90 per cent ether) extracted a cholesterol fraction which he called "readily extractable cholesterol."

Over a period of some years sporadic attempts were made in this laboratory to develop a chemical technic for extracting, in a reasonably quantitative fashion, chylomicronous fat. To date, such efforts have been of no avail, but in the course of this work it was found that extraction of plasma with benzene and with some of the other solvents noted above, under specific conditions, resulted in an entity which, in plasma from normal subjects, had the composition shown in Table I. Repeated extraction of the plasma yielded no further lipid. Because of the constancy of level of this material in a given individual, the relatively small range in a group of young normal subjects and the relative simplicity of the method, it was decided to carry out the determinations in normal and abnormal subjects under various conditions.

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MATERIALS AND METHODS

The materials are as follows: (1) solvent: reagent grade benzene; (2) a 0.2 M phosphate buffer of pH 6.0 is prepared by adding 12 ml. of 0.2 M disodium phosphate to 88 ml. of 0.2 M monopotassium phosphate. The 0.2 M disodium phosphate may be prepared by dissolving 2.84 gm. of anhydrous reagent grade in CO_2-free distilled water and making up to 100 ml. volume. The 0.2 M monopotassium phosphate may be prepared by dissolving 2.72 gm. in CO_2-free distilled water and making up to a volume of 100 ml.; (3) mechanical shaker; and (4) screw cap tubes (50 ml.), with polyethylene cone caps.

In a 50 ml. screw cap tube are placed 2 ml. of plasma and 2 ml. of phosphate buffer, and in a second tube are placed 2 ml. of water and 2 ml. of phosphate buffer; 20 ml. of solvent is then added to each tube. The tubes are tightly capped, using washed polyethylene cone caps, and shaken in a mechanical shaker for

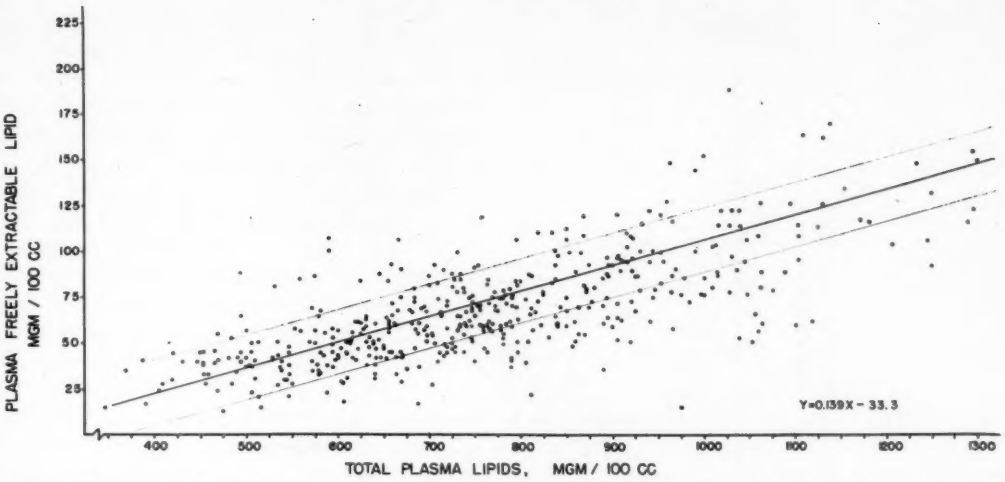

FIG. 1. Comparison of 477 freely extractable and total plasma lipid determinations in an unselected group of normal and abnormal subjects. The correlation coefficient between freely extractable and total lipid is 0.749. The least squares and limit of confidence lines apply only to values for the normal group (sixty-six subjects).

fifteen minutes. They are next centrifuged at approximately 2,000 r.p.m. for ten minutes, and 15 ml. of the supernatant is pipetted into 25 ml. volumetric flasks. The solvent is evaporated under partial vacuum at approximately 50°C., to complete dryness, and the total lipids are determined by the colorimetric method of Bragdon.[3]

In the majority of plasmas studied, in addition to freely extractable lipid, the following analyses were carried out: free and total cholesterol,[4] unesterified fatty acids,[5] phospholipids[6] and glycerides.[7]

For reasons that will be noted, if the patient has received heparin, extraction of the blood plasma for the determination of the freely extractable lipid must be carried out immediately or the plasma must be frozen. Otherwise, the blood may be kept in a refrigerator overnight.

RESULTS

Normal Plasma Values

Fasting blood was obtained from twenty-eight young male Air Force Cadets, ranging in age from eighteen to twenty-four years. These men were in "perfect" physical condition. Their diet was essentially "average American" in type. The mean value for freely extractable lipid was 47 mg. per 100 ml., with a S.E. of ±2.40 (Table I). The mean value of total lipids was 596 mg. per 100 ml., with a S.E. of ±13.73. The mean total cholesterol was 161 mg. per 100 ml., with a S.E. of ±4.28.

Plasmas from Patients with Known or Suspected Lipid Abnormalities

Fasting blood samples were obtained from a group of male and female patients. The freely

TABLE I
Fasting Plasma of the Normal Young Man

Lipid Fraction	mg./100 ml. Plasma	Per Cent of Freely Extractable Lipid
Cholesterol ester	31.0	66
Free cholesterol	3.8	8
Unesterified fatty acids	7.5 (as palmitic acid)	16
Glycerides	4.7	10
Phospholipids	0	0
Total	47.0 (±2.40)	100

Mean Total Plasma Cholesterol.....161 mg./100 ml. (±4.28)
Mean Total Plasma Lipid..596 mg./100 ml. (±13.73)

NOTE: Figures in parentheses are standard errors.

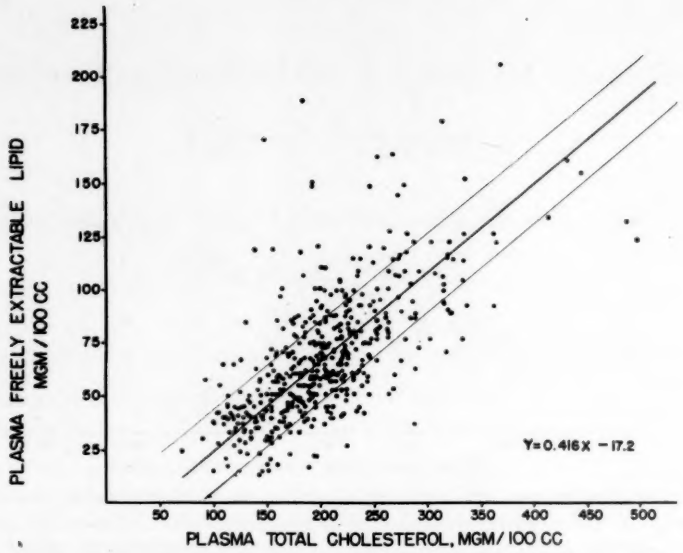

FIG. 2. Comparison of 487 freely extractable lipid and total plasma cholesterol determinations in an unselected group of normal and abnormal subjects. The correlation coefficient between total cholesterol and freely extractable lipid is 0.519. The least squares and limit of confidence lines apply only to values for the normal group (sixty-six subjects).

extractable lipid, total cholesterol and total plasma lipids were determined. A plot which shows the relation of the total lipids to the freely extractable lipid may be seen in Figure 1. The correlation coefficient is 0.749. The relationship between freely extractable lipids, and total cholesterol is shown in Figure 2. The coefficient of correlation is much lower than in the case of the total lipids (0.519). Generally speaking, the freely extractable lipid tended to be proportionately higher than the total cholesterol. In most instances where glycerides were determined, the freely extractable lipid reflected not only the level of total cholesterol but of total glycerides. Many of the patients included in these graphs had atherosclerotic involvement, with values for glycerides proportionately higher than for total cholesterol.

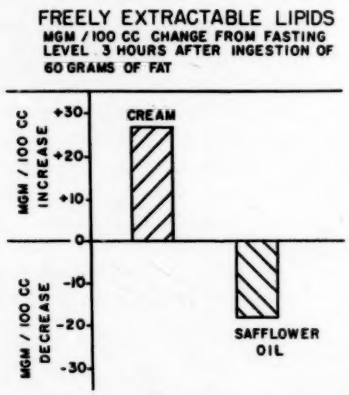

FIG. 3. Butterfat "loading" increased and safflower "loading" decreased the freely extractable lipid in the majority of a group of twenty-one subjects.

Fat Loading

After fasting blood samples were taken, a group of twenty-one patients received an emulsion containing 60 gm. of butterfat, 16 gm. of protein and 22 gm. of lactose. Three hours after the emulsion was given, a second blood sample was taken. A week or two later the same patients received an emulsion containing 60 gm. of safflower oil, 30 gm. of protein and approximately 50 gm. of lactose. Blood samples were obtained at the same intervals as before. With butterfat, the freely extractable lipid increased 27 mg. per 100 ml. above

the fasting level; with safflower oil there was a decrease of about 18 mg. per 100 ml. (Fig. 3).

With butterfat, two patients showed no change of the total plasma lipids and the others had an average increase of 123 mg. per 100 ml. Insignificant changes from the fasting level of total lipids followed ingestion of safflower oil. Plasma total cholesterol did not change significantly with either fat.

After ingestion of either butterfat or safflower oil, approximately 50 per cent of the unesterified fatty acids appears in the freely extractable lipid fraction. There was a major increase in the plasma unesterified fatty acids fifteen minutes after injection of heparin, and essentially all of the unesterified fatty acids appeared in the freely extractable lipid fraction (Fig. 4).

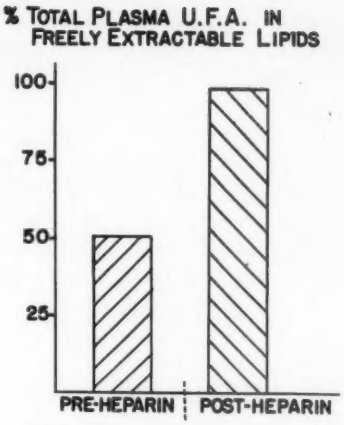

FIG. 4. In fasting plasma and after a fatty meal approximately 50 per cent of the total unesterified fatty acids appear in the freely extractable lipid. After injection of heparin, almost all the unesterified fatty acids are in this fraction. This suggests that the unesterified fatty acids are very loosely bound to protein, particularly after heparin administration.

COMPOSITION OF THE FREELY EXTRACTABLE LIPIDS IN NORMAL AND ABNORMAL PLASMAS

Normal Plasmas

The large amount of cholesterol esters and the lack of phospholipid (Table I) might suggest that the major source of the freely extractable lipid in normal subjects is the low density lipoproteins, since Avigan[8] has shown that cholesterol, but not phospholipid, may be removed from these entities without disrupting their fundamental integrity.

The 66 per cent cholesterol ester (Table I) was calculated using Bragdon's factor of 1.68, which is based on the assumption that the fatty acid in combination with the cholesterol is one with a carbon chain length of 16 to 18.

Abnormal Plasmas

Blood plasma samples from five patients whose total plasma lipids averaged 960 mg. and total cholesterol 296 mg. per 100 ml. were analyzed individually. The mean freely extractable lipid of these patients was 120 mg. per 100 ml., compared with the mean normal of 47 mg. per 100 ml. The glyceride fraction was 20 per cent, as compared to 10 per cent in the normal. Injection of heparin increased the unesterified fatty acids and decreased the glyceride (Table II).

Table III shows the pre- and postheparin composition of the freely extractable lipids in two patients with considerable elevation of glycerides and very moderate elevation of cholesterol.

TABLE II

Composition of the Freely Extractable Lipid of Five Patients with Moderately Elevated Lipids

	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. of Heparin	
Lipid Fraction	mg./ 100 ml. Plasma	Per Cent of Freely Extractable Lipids	mg./ 100 ml. Plasma	Per Cent of Freely Extractable Lipid
Cholesterol ester	73	61	71	46
Free cholesterol	15	12	14	9
Unesterified fatty acid	8	7	53	34
Glycerides	24	20	17	11
Phospholipids	0	0	0	0
Total	120	100	155	100

Mean Total Plasma Cholesterol . . . 296 mg. per 100 ml.
Mean Total Plasma Lipids . . . . . 960 mg. per 100 ml.

TABLE III

Composition of the Freely Extractable Lipid in Two Patients with Hyperlipemia and Slightly Elevated Cholesterol

Lipid Fraction	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	126	13	82	14
Free cholesterol	59	6	33	6
Unesterified fatty acids	14	1	68	12
Glycerides	601	60	285	48
Phospholipids	55	5	77	13
Sum of above	855	85	545	93
Total found	1,015	...	589	...
Unaccounted for	160	15	44	7

Mean Total Plasma Cholesterol...275 mg. per 100 ml.
Mean Total Plasma Lipids.....2,850 mg. per 100 ml.
Mean Total Plasma Glycerides..2,192 mg. per 100 ml.
Mean Total Plasma Phospholipids.295 mg. per 100 ml.

In Table IV are the findings in two patients with much greater elevation of cholesterol and considerably greater elevation of total lipids. Percentage composition of the freely extractable lipids in the two groups is not too dissimilar. There are major differences between the two groups in terms of response to heparin. The interpretation of these differences will be considered at another time. The absence of phospholipids in the freely extractable lipids in normal and slightly abnormal plasma, and the presence of phospholipids in the freely extractable lipid of hyperlipemic plasma is of interest.

Blood plasma samples of eight patients without obvious lipid abnormality, who had received oral butterfat emulsion followed by intravenous injection of heparin were analyzed separately. The average values for this group are shown in Table V. The composition of the freely extractable lipid fraction was similar to that reported for the normal fasting subjects,

TABLE IV

Composition of the Freely Extractable Lipid in Two Patients with Hyperlipemia and Hypercholesterolemia

Lipid Fraction	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	485	14	567	16
Free cholesterol	220	7	179	5
Unesterified fatty acids	31	1	143	4
Glycerides	2,430	70	2,421	70
Phospholipids	140	4	114	3
Sum of above	3,306	96	3,424	98
Total found	3,440	...	3,500	...
Unaccounted for	134	4	76	2

Mean Total Plasma Cholesterol...912 mg. per 100 ml.
Mean Total Plasma Lipids.....4,362 mg. per 100 ml.

TABLE V

Composition of the Freely Extractable Lipid in Eight Patients Three Hours after Oral Administration of an Emulsion Containing 60 Gm. of Butterfat

Lipid Fraction	3 Hours after Fatty Meal		15 Min. after Intravenous Administration of 50 mg. of Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	51.4	56	38.2	29
Free cholesterol	10.8	12	8.6	7
Unesterified fatty acids	15.0	17	74.0	56
Glycerides	14.0	15	10.0	8
Phospholipids	0	0	0	0
Total	91.2	100	130.8	100

Mean Total Plasma Cholesterol...223 mg. per 100 ml.
Mean Total Plasma Lipids.....828 mg. per 100 ml.
Mean Total Plasma Glycerides....254 mg. per 100 ml.
Mean Total Plasma Phospholipids 254 mg. per 100 ml.

although the total freely extractable lipid was nearly twice that found in the fasting state. Intravenous administration of heparin in this group caused an actual, as well as a percentual, lowering of esterified cholesterol and of glycerides, and an increase of unesterified fatty acids.

In thirteen patients without gross lipid abnormality who received safflower oil, the composition of the freely extractable lipid before and after injection of heparin was similar to the composition after ingestion of butterfat.

The biochemical and clinical significance of these findings will be the subject of a later report.

SUMMARY AND CONCLUSIONS

The freely extractable lipid is that plasma lipid fraction which can be extracted with benzene under certain specified conditions. The freely extractable lipid level in fasting young normal males averages 47 mg. per 100 ml. The qualitative-quantitative composition of the freely extractable lipid in normal subjects is remarkably constant. Major deviations from normal occur in some patients with atherosclerosis and in all patients in a hyperlipemic state.

In blood plasma with total lipid levels under 1,500 mg. per 100 ml., the bulk of the freely extractable lipid consists of cholesterol esters with a small amount of free cholesterol, glycerides and free fatty acids and no phospholipids. Blood plasma with total lipids in excess of 2,000 mg. per 100 ml. shows a larger proportion of glycerides and some phospholipids in the freely extractable lipid.

Approximately 50 per cent of the unesterified fatty acids of the plasma appear in the freely extractable lipids before heparin is given and nearly all of the unesterified fatty acids appear in the freely extractable lipid after injection of heparin. This suggests that much of the unesterified fatty acid is loosely bound to protein. Administration of heparin also influences other lipid components of the freely extractable lipid.

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Investigation of Serum Lipoprotein Metabolism in Man with I^{131}- Labeled Triolein

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THE present investigation was undertaken to study the role of the several lipoprotein components of human serum in the transport of dietary fat. Labeled fatty acids in the form of I^{131} triolein were fed to normal subjects and patients with various blood lipid abnormalities in order to study the nature of these deviations from normal and their effects on lipid transport.

METHODS

Following an overnight fast, 30 ml. of venous blood was obtained from each subject and was permitted to clot. One gm. of sodium iodide was then administered intravenously to prevent thyroid uptake of I^{131} and conversion to protein-bound iodine. The subject was then fed a standard breakfast containing 60 gm. of carbohydrate, 10 gm. of protein and 20 gm. of fat including I^{131} triolein (Raolein,® Abbott). The labeled lipid was previously emulsified with a phosphatide-glycerol (1:10 w/w) mixture (30 ml. mixture plus 1 to 2 ml. Raolein plus 300 ml. water in Waring Blendor) and 50 μc. aliquots added to milk. At intervals of two, four, seven and twenty-four hours, 30 ml. samples of venous blood were drawn, permitted to clot at 4°C. and the sera separated by centrifugation at 500 × G for twenty minutes.

Lipoprotein fractions were separated by a density gradient ultracentrifugation procedure.[1] Five ml. portions of the serum were placed in lusteroid centrifuge tubes of 13.5 ml. capacity, overlayed with 4 ml. of 0.15 M sodium chloride and centrifuged for thirty minutes at 9,300 × G in the Spinco Model L ultracentrifuge. The chylomicra formed a turbid band at the top of the saline layer and were separated from the infranatant serum with a tube cutter. Dark-field examination indicated essentially complete removal of visible particles of chylomicron from the serum. This serum was then layered over 5.5 ml. of 2 M sodium chloride in another lusteroid tube, overlayed with 0.15 M sodium chloride to fill the tube, and centrifuged at 100,000 × G for eighteen hours. The S_f 10–400 lipoproteins were concentrated in a turbid band at the top of the tube, the S_f 3–9 lipoproteins formed a clear orange-yellow band in the center, while the high density lipoproteins plus the remaining serum proteins sedimented to the bottom of the tube. The lipoprotein fractions were separated with the tube cutter. The fractions from the zero-hour specimen were transferred to volumetric flasks and the lipids extracted with ethanol-ether mixture (3:1 v/v) for cholesterol and phospholipid analysis. The two, four, seven and twenty-four-hour specimen fractions were quantitatively transferred to test tubes with minimal amounts of 0.15 M saline and the final volume measured. Two ml. aliquots of the fractions as well as of the original sera were then treated to remove inorganic I^{131} by adding

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TABLE I

Per Cent of Total Dose of I^{131} Administered per Liter Serum at Various Intervals after I^{131}-Triolein Test Meal in Normal, Hypercholesterolemic (Elevated S_f 3–9) and Hyperlipemic (Elevated S_f 10–400) Subjects

Subject	Total Serum I^{131} Per Cent Dose/Liter				Serum Lipid I^{131} Per Cent Dose/Liter				Lipid I^{131}/Total I^{131}			
	2 hr.	4 hr.	7 hr.	24 hr.	2 hr.	4 hr.	7 hr.	24 hr.	2 hr.	4 hr.	7 hr.	24 hr.
Normal												
D. M.	1.53	4.60	3.28	0.88	0.81	3.40	1.70	0.15	0.53	0.74	0.52	0.17
R. K.	2.31	4.35	2.52	0.50	0.81	2.96	1.16	0.09	0.35	0.68	0.46	0.18
M. S.	5.71	6.48	4.30	0.78	3.37	3.43	0.73	0.07	0.59	0.53	0.17	0.09
L. P.	3.75	4.96	4.15	1.04	1.35	1.94	1.37	0.05	0.36	0.39	0.33	0.05
R. F.	3.21	3.55	2.53	0.90	0.38	0.90	0.32	0.04	0.12	0.25	0.13	0.04
Elevated S_f 3–9												
G. G. (Idiopathic hypercholesterolemia)	4.86	6.55	2.85	0.60	3.20	4.45	0.57	0.11	0.66	0.68	0.20	0.18
C. K. (Nephrosis)	3.07	5.95	3.91	0.74	1.78	4.23	1.72	0.06	0.58	0.71	0.44	0.08
P. S. (Myxedema)	1.18	2.70	1.82	0.67	0.53	1.70	0.84	0.12	0.45	0.63	0.46	0.18
W. S. (Myxedema)	2.67	7.20	8.40	2.39	1.14	4.78	6.36	0.58	0.43	0.67	0.76	0.24
W. H. (Biliary cirrhosis)	3.40	5.04	5.36	1.71	1.98	3.18	3.46	0.27	0.58	0.63	0.64	0.16
Elevated S_f 10–400												
H. A. (Xanthoma tuberosum)	1.74	5.65	6.06	5.14	0.92	4.63	5.22	4.21	0.53	0.82	0.86	0.82
H. P. (Hyperlipemia)	1.02	2.63	3.48	6.74	0.40	2.27	2.86	6.20	0.39	0.86	0.82	0.92
W. S. (Diabetes; hyperlipemia)	4.75	5.74	5.12	2.19	3.60	4.31	3.72	1.41	0.76	0.75	0.73	0.65
L. S. (Xanthoma tuberosum)	0.74	4.40	8.24	2.38	0.18	1.46	4.11	1.25	0.24	0.33	0.50	0.53

0.1 gm. of Dowex® 2 × 10 anion exchange resin in the chloride form, agitating gently for several minutes to ensure complete removal and spinning at low speed in a clinical centrifuge to separate the resin. One ml. aliquots of the supernatant were then assayed for radioactivity as were 1 ml. samples of the original sera. The results were expressed as per cent of dose per liter for total and lipid radioactivity in whole serum and lipid radioactivity in the chylomicron, S_f 10–400, S_f 3–9 and high density lipoprotein fractions.

RESULTS

Some representative values obtained from normal subjects and patients with abnormal blood lipid patterns are given in Table I. It may be seen that in the normal subjects peak levels of lipid radioactivity as well as total radioactivity always occurs before seven hours. In addition the lipid radioactivity at the end of twenty-four hours is negligible even though the total radioactivity may be appreciable. This is expressed another way in the last column which shows that the lipid fraction of the total radioactivity after twenty-four hours is always less than 20 per cent and usually considerably less than this. Figure 1 shows a typical distribution of the lipid radioactivity among the various lipoprotein fractions at different times. The labeled lipid appears mainly in the S_f 10–400 and chylomicron fractions.

The second group in Table I consists of patients with elevated S_f 3–9 lipoprotein levels (the hypercholesterolemic states). As a group they do not appear to handle the labeled lipid much differently than the normal subjects,

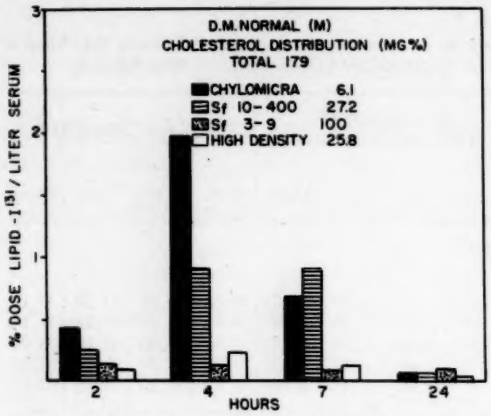

FIG. 1. Distribution of I^{131}-labeled lipid in serum lipoproteins after I^{131}-triolein meal in a normal subject.

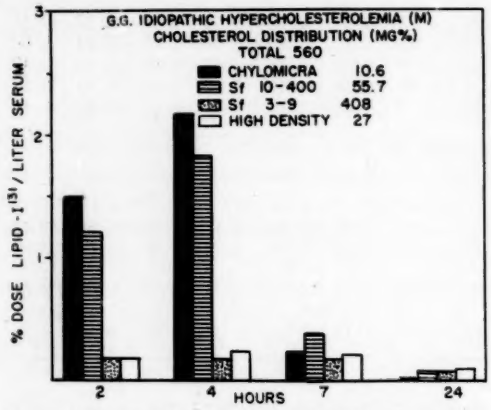

FIG. 2. Distribution of I^{131}-labeled lipid in serum lipoproteins after I^{131}-triolein meal in a patient with hypercholesterolemia.

although the patients with myxedema show a tendency to delayed removal of the labeled lipid. This is particularly evident in patient W. S. who shows a delayed peak (seven hours) as well as a somewhat higher than normal lipid I^{131} fraction after twenty-four hours (24 per cent). Figure 2 shows the transport pattern of a patient (G. G.) with atherosclerosis (post-myocardial infarction) and hypercholesterolemia. In spite of a total serum cholesterol of 560 mg. per 100 ml. and markedly elevated S_f 3–9 fraction, his handling of dietary lipid as measured by this test appears normal. Figure 3 illustrates the pattern of one of the patients with myxedema (P. S.). Aside from the suggestion of a slight delay in clearing, the over-all pattern appears normal.

The last group in Table I contains representative examples of patients with elevated S_f 10–400 lipoprotein levels (the hyperlipemic states). The transport patterns here are all grossly abnormal. Peak labeled lipid levels are usually beyond the four-hour period. Even more striking is the high percentage of the total radioactivity which is still in the lipid fraction after twenty-four hours. This is always over 50 per cent and frequently approaches 90 per cent. Figure 4 shows the pattern obtained from a thirty-seven year old white man (H. A.) with lipemic serum and xanthoma tuberosum. His serum lipid I^{131} at twenty-four hours was still 81 per cent of the peak value and the lipid portion of the total radioactivity at twenty-four hours was 82 per cent of the total. Figure 5 illustrates an even more extreme defect in clearing ability. This patient (H. P.) had a total serum cholesterol of 1212 mg. per 100 ml., 705 mg. of which was found in the S_f 10–400 fraction and 346 mg. in the chylomicron fraction. The amounts in the S_f 3–9 (108 mg. per 100 ml.) and high density (26 mg. per 100 ml.) were within normal limits. The total lipid I^{131} at twenty-four hours was more than twice that at seven hours, and this represented ninety-two per cent of the total I^{131} present in the serum.

COMMENTS

Previous studies employing lipoproteins labeled in the protein moiety have indicated that S_f 10–400 and S_f 2–9 lipoproteins are related to each other as precursor and product.[2] In addition, these studies demonstrated that in patients with nephrosis and hyperlipemia the elevation in S_f 10–400 lipoproteins could be explained at least in part as resulting from a delay in the transformation of this class to the S_f 3–9 class. Immunochemical studies[3] also indicate a close relationship between the two classes of lipoproteins. The fate of the chylomicrons and their relationship to other serum lipoproteins have been more difficult to elucidate, but it appears to be established that the protein moieties of the chylomicra and the high density lipoproteins are similar if not identical.[4]

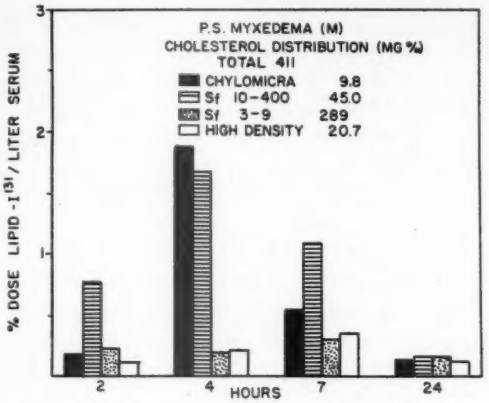

FIG. 3. Distribution of I^{131}-labeled lipid in serum lipoproteins after I^{131}-triolein meal in a patient with thyroid deficiency and hypercholesterolemia.

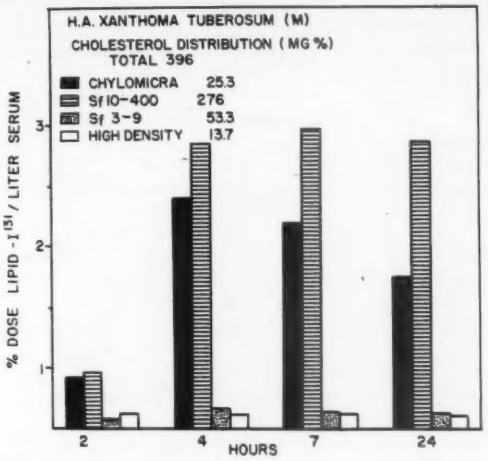

FIG. 4. Distribution of I^{131}-labeled lipid in serum lipoproteins after I^{131}-triolein meal in a patient with hyperlipemia and xanthoma tuberosum.

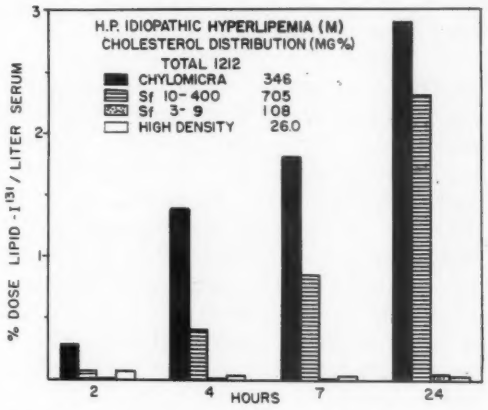

FIG. 5. Distribution of I^{131}-labeled lipid in serum lipoproteins after I^{131}-triolein meal in a patient with idiopathic hyperlipemia.

We have observed[5] that the intravenous infusion of fat emulsions results in a decrease in high density lipoprotein cholesterol corresponding to an increase in the "chylomicron" fraction. The disappearance of these "chylomicrons" coincides with an increase in the S_f 10–400 lipoprotein fraction. It would appear that the chylomicrons and high density lipoproteins constitute one system and the S_f 10–400 and S_f 3–9 lipoproteins a distinct but parallel system for transporting triglycerides in the blood.

We can picture the chylomicrons as the initial transport vehicle for the transport of exogenous fat in the blood. These are rapidly cleared from the blood, presumably in the reticuloendothelial system, and triglyceride reincorporated into newly synthesized S_f 10–400 molecules in the liver.[6] The latter molecules can be considered as secondary transport vehicles for exogenous fat and in addition as vehicles for the transport of endogenous triglyceride. The S_f 3–9 molecules appear to be synthesized directly by the liver in the absence of a lipid load, as well as being formed from an unloading of S_f 10–400 molecules. In the presence of an exogenous lipid load resulting from the clearing of chylomicrons after a fat meal or under conditions where endogenous non-esterified fatty acids are elevated as in diabetes mellitus, excess fatty acid is incorporated as triglyceride to form S_f 10–400 molecules rather than S_f 3–9 molecules. These relationships are shown schematically in Figure 6.

The use of I^{131}-labeled fatty acids to study mechanisms of lipid transport is subject to criticism. Van Handel and Zilversmit[7] for example, have shown in experiments with dogs that the specific activity of plasma triglycerides after oral administration of mixtures of I^{131} triolein with various fats and oils was always less than that of the fed triglyceride mixture. The same was true of triglycerides of the thoracic

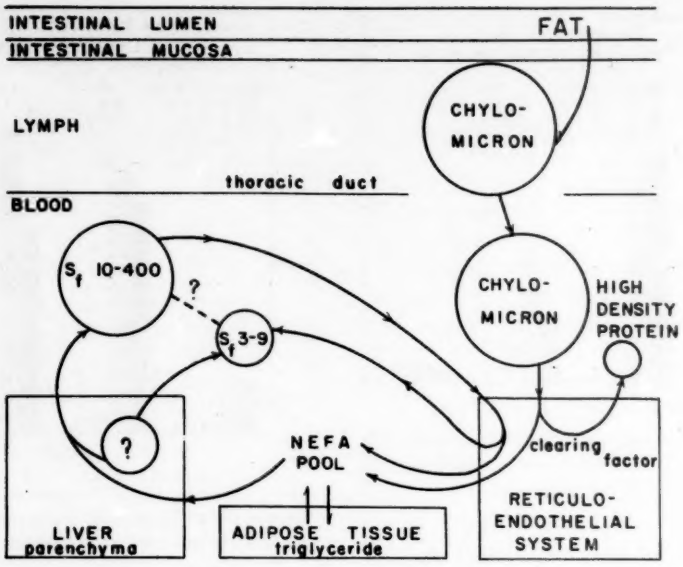

FIG. 6. Hypothetical scheme showing transport of dietary triglyceride via the blood lipoprotein system.

duct lymph in the rat. That this was not a dilution effect due to endogenous triglyceride was shown by the fact that the specific activity of C^{14}-labeled fatty acid containing triglycerides was the same in the duct lymph as in the meal. Thus, there is a difference in the appearance in lymph and blood of I^{131}-labeled and unlabeled triglycerides. This difference might reflect a difference in rate of absorption or might indicate a preferential catabolism of the I^{131} lipid somewhere between the intestinal lumen and the thoracic duct.

On the other hand, these same authors showed that intravenously administered I^{131}-labeled fat emulsions in dogs maintained a constant specific activity throughout a two-hour period during which most of the infused lipid disappeared from the circulation. It would thus appear that the rate of removal of I^{131}-labeled triglyceride from the circulation is a valid measure of the rate of removal of unlabeled triglyceride. It must be pointed out however, that since the level of blood lipids at any given time is the resultant of the processes of absorption and clearing, the actual values reported should not be interpreted as indicating those which would be found with unlabeled or C^{14}-labeled lipid. On the other hand, this label, apart from ease in handling, offers certain advantages over chemical analysis for triglyceride since the twenty-four–hour period following a given meal can be studied without interfering with or interference from the normal spacing or content of meals throughout the day. It also has the advantage over C^{14}-labeling in that once metabolized there is no reincorporation of the label in either lipid or other substances.

With these considerations in mind it appears from the results presented that I^{131}-labeled triglyceride is a useful tool in the study of fat transport in abnormal as compared with normal blood lipid states. The dynamics of I^{131}-lipid transport reflects the defects in triglyceride clearing in hyperlipemic states and also the essentially normal transport of triglyceride in hypercholesterolemic states.

It should be pointed out that approximately 90 per cent of the labeled serum lipid following the ingestion of I^{131}-labeled triolein is present as triglyceride, and the remainder mostly as diglyceride. No label is found in the phospholipid and cholesterol ester fractions as separated on silicic acid columns.[8] It is also of interest

to note that in contrast to phospholipid[9] and cholesterol,[10] which exchange rapidly *in vitro* between the various lipoprotein species, I^{131}-labeled triglyceride, in addition to being associated primarily with chylomicron and S_f 10–400 species, does not exchange appreciably between these two fractions *in vitro*.[11]

SUMMARY

I^{131}-labeled triolein was fed to subjects with normal and abnormal blood lipid patterns in order to study the role of the various serum lipoproteins in lipid transport. In all subjects labeled lipid appeared almost exclusively in the chylomicron and S_f 10–400 lipoprotein fractions. In the normal and hypercholesterolemic (elevated S_f 3–9) subjects the labeled lipid peaked early and was essentially cleared from the blood within twenty-four hours. Patients with hyperlipemia (elevated S_f 10–400) exhibited a delayed peaking of the I^{131}-lipid level which was still considerably elevated after twenty-four hours. The results are interpreted in terms of a hypothetical scheme for lipid transport. The validity of results obtained with I^{131}-labeled lipid is discussed.

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Effects of Essential Fatty Acid Deficiency and X-Radiation on the Plasma Cholesteryl Ester Spectrum of the Rat

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THE PRESENT study was motivated by curiosity concerning possible changes in the fatty acid spectrum of plasma cholesteryl esters accompanying such phenomena as the development of overt symptoms of deficiency in the mouse with chronic deficiency of fat following exposure to x-radiation.[1] It seemed reasonably certain that the animal's economy of polyunsaturated fatty acids is altered under such circumstances, and since the plasma cholesteryl esters are easily accessible, are known to be rich in acids of this type and are suspected of being involved in their transport, these lipids were an obvious choice for initial scrutiny.

METHODS

Adult male Sprague-Dawley rats were employed, pooled plasma from groups of fifteen to twenty-four animals was extracted with acetone, cholesteryl esters were isolated by an adaptation of the silicic acid adsorption chromatographic technic of Fillerup and Mead[2] and mixtures of fatty acids released from the esters by saponification were analyzed by reversed phase chromatography before and after catalytic hydrogenation.[3]

RESULTS

Table I presents a selection of analyses obtained from groups of animals with these histories: group I-C, reared on Rockland rat diet, maintained on synthetic fat free diet[1] supplemented with 4 per cent corn oil for fifteen days before sacrifice; group I-FF, the same diet except for omission of the corn oil supplement; group II-FF, raised on a fat free diet from weaning to cessation of growth, fasted two days and fed about 20 per cent of normal *ad libitum* intake on day before sacrifice; group II-FFX, the same diet as group II-FF, but exposed to 600 r, 250 kv. x-radiation of the whole body three days before sacrifice.

Seven different groups of animals yielded analyses, of which group I-C is typical, with arachidonic comprising about two-thirds of the total and the bulk roughly equal amounts of palmitic, oleic and linoleic acids. Under conditions employed in these studies, this fatty acid spectrum was not greatly altered except when ingestion of linoleic acid was restricted (see following paragraph). Although the normal rat spectrum reported here has been observed repeatedly and was checked in one instance by gas-liquid chromatography, other presumably comparable analyses which have appeared in the literature [4,5] are difficult to reconcile with it. The ratio of arachidonic to linoleic acid (5 ± 1) in the rat is much higher than in the dog (0.48),[6] in the cow (0.17)[7] or in man (0.17).[8]

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Changes in the fatty acid composition of the lipids of animals maintained on diets deficient in linoleic acid (additional examples of which have been reported by Dr. Ralph Holman at this Conference) have been rationalized recently by Fulco and Mead[9] in terms of abortive transformations wrought on acids such as palmitoleic and oleic, which the animal can synthesize *de novo*, instead of on the normally utilized but unsynthesizable linoleic acid. A grotesque example of such changes is afforded by the plasma cholesteryl ester spectrum of the rat raised from weaning to cessation of growth on a diet free of linoleic acid (group II-FF): while palmitic and linoleic acids are essentially absent and arachidonic (or, more probably, an isomeric eicosatetraenoic derived from palmitoleic acid) occurs in very minor amount, oleic is now the major acid and two new components, palmitoleic and C_{20}-trienoic, are present in important quantities. Remarkably, a trend in this direction is readily discernible in the normal animal in as little as fifteen days after withholding linoleic acid from the diet (see group I-FF). (Here again, earlier analyses[4,5] are at odds with these observations.)

Except for a possibly significant slight increase in the proportion of arachidonic acid, x-irradiation of the whole body produces little change in the plasma cholesteryl ester spectrum of the animal (as reported earlier by Deuel and co-workers[10]) unless it be truly deficient in fat (as by rearing from weaning on a diet free of linoleic acid, group II-FFX), in which case a dramatic general increase in amounts of polyunsaturated fatty acids at the expense of the monoenoic components is observed.

TABLE I
Rat Plasma Cholesteryl Ester Compositions
(Per Cent of Total)

Acid	Group			
	I-C	I-FF	II-FF	II-FFX
Palmitic	6.2	8.9	0	0
Palmitoleic	0	11.4	13.4	5.4
Oleic	8.2	15.7	64.5	38.3
Linoleic	18.0	11.2	0	4.5
C_{20}-trienoic	0	7.1	18.2	32.5
Arachidonic	67.6	45.9	3.9	19.0

COMMENTS

These exploratory studies bear several interesting implications which would seem to warrent emphasis. First, the apparent constancy of the arachidonic-linoleic acid ratio even when supplies of the former are at a premium, suggests that linoleic may have some function in addition to that of serving as the obligate precursor of arachidonic acid. Second, the absence of evidence of stimulation of the production of pseudo essential fatty acids (i.e., those derived from oleic or palmitoleic acids) in the normal animal maintained post-radiation on a diet free of linoleic acid may evidence the availability for regenerative processes of true essential fatty acids released by disintegrating irreparable cells. Third, the appearance of eicosatrienoic acid in the plasma cholesteryl esters shortly after withdrawal of linoleic acid from the diet indicates that the linoleic-arachidonic transformation depends heavily, if not exclusively, on the dietary supply of linoleic acid, the rather substantial quantities of linoleic acid normally present in the tissues (including the blood) being not readily available for this presumably vital process.

SUMMARY

Exploratory studies of the serum cholesteryl ester spectrum of the rat indicate that when linoleic acid is withdrawn from the diet, the normally predominant arachidonate and linoleate components begin to be replaced rather quickly by other unsaturated fatty acid moieties which do not require linoleate as an obligatory precursor. Although exposure of the animal to 600 r x-radiation does not greatly effect the normal spectrum, such a physiologic insult dramatically aggrevates the abnormality of the spectrum of the animal with deficiency of fat.

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Effect of Dietary Fat on Synthesis and Degradation of Cholesterol by the Liver

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THE findings that the addition of unsaturated fat to the diet will lower the serum cholesterol levels of adults[1,2] and even of infants[3] have stimulated considerable work in this area, and these effects are now amply documented. How the unsaturated fat works is an important question, and the answers are still being gathered. In man there is evidence of increased excretion of cholesterol and bile acids.[4–6] A similar effect has been observed in rats, although in these animals there also appears to be increased adsorption of cholesterol.[7]

It is only natural that attention should turn to the intermediary metabolism of cholesterol under the influence of different fats. Several laboratories have investigated the biosynthesis of cholesterol as affected by dietary fat, and the results show an increased synthesis by animals fed unsaturated fat. Thus, Mukherjee and Alfin-Slater[8] compared the effects of cottonseed oil, hydrogenated coconut oil, fat-free diet and fat-free diet plus methol linoleate. Using the first diet as a control, they found that incorporation of acetate-C^{14} into rat liver cholesterol was, after sixteen weeks on the diet, 7.4, 12.2 and 100 per cent respectively for the other three diets. Wood and Migicovsky[9] compared rapeseed oil, corn oil and coconut oil and found the first two to enhance cholesterol synthesis from acetate *in vivo*. Avigan and Steinberg[10] compared coconut oil and corn oil, using both acetate-C^{14} and T_2O as substrates and found that the unsaturated diet enhanced cholesterol synthesis from either precursor. Linazasoro et al.[11] found no differences in cholesterol biosynthesis, but their feedings were carried out for three days whereas the other authors cited carried out their feedings for at least a week. They did, however, find considerably more synthesis in the fat-fed animals than in control animals kept on a fat-free diet.

Drs. Whitehouse, Staple and I have been investigating the oxidation of the terminal carbon atoms of cholesterol by the liver mitochondria of rats fed diets rich in corn oil or a commercial shortening (Crisco). Both fats were fed as 20 per cent of the diet. The preparation of the enzyme system has been described recently.[12] We find that whether we use livers of male rats, female rats or pooled livers from male and female rats, the mitochondria of the Crisco-fed rats oxidize cholesterol-26-C^{14} to $C^{14}O_2$ at a faster rate than do mitochondria from rats fed corn oil. Rats maintained on a normal diet were used as control animals. The results are summarized in Table 1.

The results were consistent in each separate experiment (i.e., more oxidation with shortening than with corn oil) but the variation between experiments precludes any speculation concerning an effect of differences in sex.

Liver fat from rats on the unsaturated fat diet, when added to normal mitochondrial preparations, inhibited oxidation of cholesterol-26-C^{14} to the same extent as did liver fat from rats on the shortening diet. The

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TABLE I

Effect of Dietary Fat on Oxidation of Cholesterol-26-C^{14} by Rat Liver Mitochondria

Animals	No. Experiments	Per Cent Oxidation (Average)*		
		Control	Corn Oil	Crisco
Males	4	12.1	6.1	15.8
Females	5	8.7	7.4	14.4
Mixed	3	14.1	9.9	14.2

* All values corrected for equivalent amounts of mitochondria (mg. N).

dietary effect was not observed when the same enzyme systems were incubated with pyruvate-2-C^{14}.

We are continuing our experiments in this area in an effort to elucidate our results.

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The Metabolism of the Polyunsaturated Fatty Acids

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FROM time to time during the past few years current knowledge of the metabolism of the polyunsaturated acids has been reviewed. Each time there have been included bits of information gathered since the last review which bring us somewhat nearer our goal: the complete elucidation of the formation and fate of these acids. Although it cannot truthfully be said that we have achieved this goal, still, with a little speculation, we can at last begin to see the whole picture rather clearly.

Most of the early evidence on the transformations of the polyunsaturated acids was obtained with the technic of alkaline isomerization. This technic gave an indication of the alterations in the unsaturated centers of the fatty acids of various tissues but gave little or no information on the alterations of the fatty acids themselves or their transformation to one another. However, interesting and provocative evidence was obtained.

For example, it was found that when a dienoic acid (linoleic) was fed to rats on a fat-free diet, the body fatty acids showed an increase in diene and tetraene;[1,2] when a trienoic acid (linolenic) was fed, there was an increase in triene, pentaene and hexaene acids in the body fat;[2,3] when the fat-free diet was continued for some time, there was an increase in triene.[1,4] In starting our studies of the metabolism of essential fatty acids we addressed ourselves principally to the interpretation of these observations.

The first experiment, which was chosen both for simplicity and because it was basic to the succeeding experiments, involved injection of carboxy-labeled acetate into young rats.[5] The organ and depot fat of these rats yielded fatty acids which were separated by crystallization at low temperature into saturated and unsaturated fractions. From the latter were obtained, by precipitation of the polybromides and chromatographic separation on a rubber column, the linoleic and (somewhat impure) arachidonic acids. The linoleic acid was found to contain essentially no activity. One-step degradation of the arachidonic acid revealed that essentially the activity resided in the carboxy group.

From these results, two conclusions were drawn. First, linoleic acid is not synthesized from acetate by the rat; second, arachidonic acid is synthesized from acetate and an exogenous C_{18} precursor.

The second experiment was designed to confirm our supposition that this exogenous precursor was indeed linoleic acid.[6] Carboxy-labeled methyl linoleate was fed to mature rats, and, after four hours, the rats were killed and the polyunsaturated acids were again isolated as the bromides and separated chromatographically, this time on the reversed-phase column of Howard and Martin. The arachidonic acid obtained from this column had been hydrogenated to arachidic acid during the separation procedure. This acid was degraded stepwise to obtain the first three carbon atoms (as benzoic acid) and the remaining seventeen

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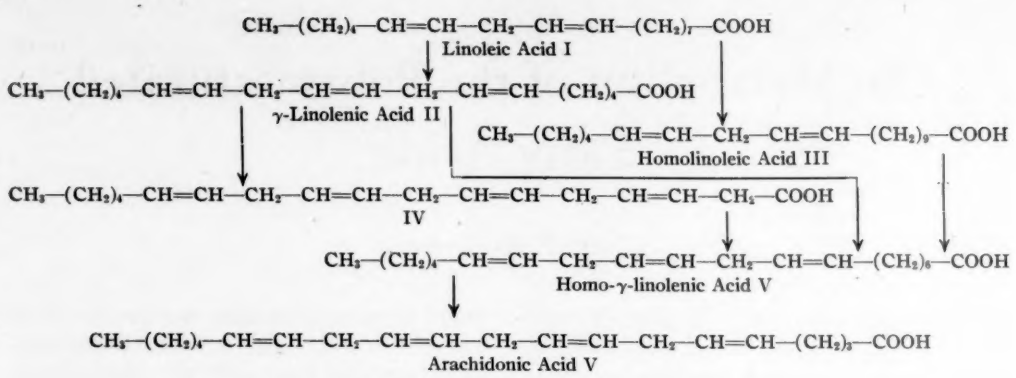


FIG. 1. Possible steps in synthesis of arachidonic acid.

carbons. The distribution of activity in the original arachidonic acid follows:

	$C_{17}H_{35}$—CH_2—CH_2—COOH			
% of total activity	0	24.5	0.7	74.7

Several conclusions were drawn from this experiment. First, since the terminal eighteen carbons have the same distribution of activity as the fed (carboxy-labeled) linoleic acid, the postulated eighteen-carbon exogenous precursor of arachidonic acid is indeed linoleic acid. Second, as was predicted from the results of the first experiment, acetate derived from catabolism of the linoleic acid contributed activity to the carboxy group of arachidonic acid. Third, the relative activity of the carboxy group of arachidonic acid is greater than was expected. This last observation will be reemphasized later.

TABLE I

Activities of Fatty Acids from Rats Fed γ-Linolenate

Fraction	Acid Component	Specific Activity (disintegrations per second per mg.)
F-1	Palmitic	1.9
	Stearic	2.5
F-2	Oleic	0.37
	Linoleic	0.63
F-3	Palmitoleic	0.45
	Linoleic (contaminated by γ-linolenic)	50.7
	Arachidonic	785
	Docosapentaenoic and docosahexaenoic	14.9

The knowledge that arachidonic acid is formed by addition of acetate to linoleic acid did not completely elucidate the biosynthetic pathway, since three steps are involved (two dehydrogenations and a chain-lengthening) and their sequence was not revealed by the experiments previously described. The possible steps in this synthesis are illustrated in Figure 1.

The choice of pathway to follow was made on the basis of the experiment of Karrer and Koenig,[7] who found that homolinoleic acid did not serve as an essential fatty acid in rats and of Thomasson,[8] who found it to possess only 40 per cent of the activity of linoleic acid in prevention of essential fatty acid deficiency. Since an intermediate in a biosynthetic scheme should possess at least the biological activity of its immediate precursor, the pathway through γ-linolenic acid was favored and was chosen for further investigation. This acid was obtained from the seeds of Oenothera Lamarckiana, a relative of the evening primrose, and was labeled in the carboxy group and fed to rats.[9] The arachidonic acid derived from their organ and depot fats was separated by crystallization at low temperature and was degraded stepwise as before. Table I gives the activity of various fatty acids from these animals.

Several points are noteworthy. First, by far the most active acid is arachidonic, indicating the direct and immediate transformation

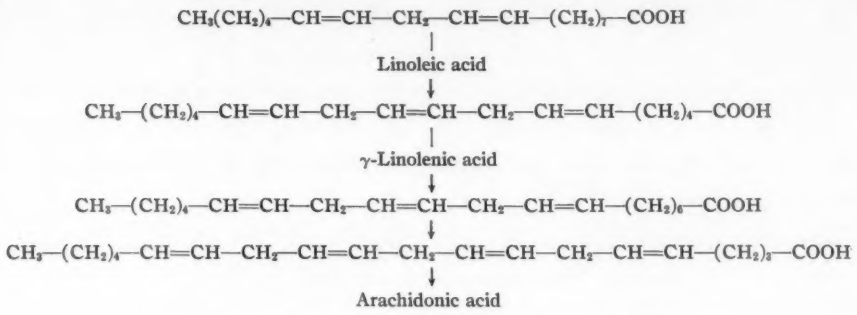


FIG. 2. Proposed scheme for pathway of linoleic to arachidonic acid.

of γ-linolenic acid to arachidonic acid. Second, an impurity in the linoleic acid from fraction F-3 (soluble in acetone at −60°C.) contributed some activity to it as shown by the very low true activity of linoleic acid as derived from F-2 (soluble at −20°C., insoluble at −60°C.). This impurity is evidently largely the fed γ-linoleic acid, which, considering its very high activity, must have been present in vanishingly small amounts. Third, the fact that the saturated acids, palmitic and stearic, are more active than their unsaturated counterparts, palmitoleic, oleic and linoleic acids, argues against hydrogenation as a significant pathway in fatty acid metabolism.

The distribution of activity in the arachidonic acid was as follows:

	$C_{17}H_{35}$—CH_2—CH_2—COOH			
% of total activity	1.2	97.4	0.3	1.1

The very high activity in the third carbon confirms the rapid and complete conversion of γ-linolenic acid to arachidonic acid. The low activity in the first carbon and the low but definite activity beyond carbon 3 will be discussed subsequently.

It thus appears that the preferred pathway from linoleic to arachidonic acid lies through γ-linolenic acid. A proposed scheme for this pathway is shown in Figure 2.

The next-to-last step (conversion of homo γ-linolenic acid to arachidonic) is now under investigation.

In a similar experiment, carboxy-labeled linolenic acid was fed, and the twenty-carbon polyunsaturated fatty acids were isolated from the organ and depot fats of the rats.[10] Degradation of this acid revealed the distribution of activity as follows:

	$C_{19}H_{33}$—CH_2—CH_2—CH_2—COOH				
% of total activity	1.05	3.8	60.2	7.8	27.2

It will be noted, first, that this distribution is intermediate between those of the previous examples and second, that there is considerable activity beyond the third carbon. The activity in the even carbons cannot be explained as yet.

The question arose as to whether or not the twenty-carbon active acid responsible for these data was indeed arachidonic. Several pieces of evidence indicate that it was not. First, a rough chromatographic separation revealed that the most active acid did not follow the distribution of arachidonic acid, but of eicosapentaenoic acid. Second, alkaline isomerization showed an increase in pentaene and hexane. Third, separate experiments, in which linolenate was fed to fat-deficient rats revealed that a docosapentaenoic acid very quickly appeared in their organ and depot fats but not in those of animals fed linoleic acid.[11] It was also noted in these last experiments that four hours after feeding linolenic acid, none could be found in the rats' tissues. Thus it appears that linolenic acid goes directly and rapidly to eicosapentaenoic and docosapentaenoic acids and, possibly, to a docosahexaenoic acid. A

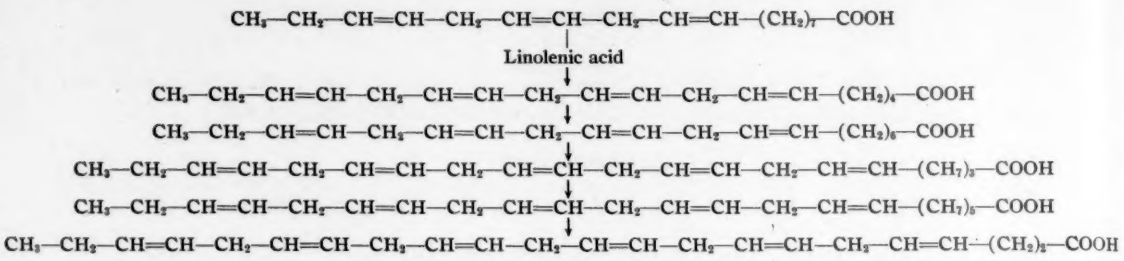


FIG. 3. Transformation of linolenic acid. See text.

possible pathway for such a transformation is shown in Figure 3.

Thus, these experiments have explained the first two alkaline isomerization findings. The third, formation of trienoic acid in animals on a fat-free diet, has also been investigated.

This acid was first isolated as the hexabromide from fat-deficient rats and was ozonized and identified by paper chromatography of the dibasic acid (glutaric) to be revealed as 5:8:11-eicosatrienoic acid[12] as shown in Figure 4.

and, in four hours, their organ and depot fats were removed and converted to fatty acids. The polyunsaturated acids were obtained by crystallization at low temperature and a C_{20} concentrate was derived from these by gas chromatography. From this concentrate was derived, by reversed-phase chromatography, an eicosatrienoic fraction. A portion of this was oxidized with permanganate to a mixture of mono- and dicarboxylic acids and a sample of oleic acid derived from these same animals was

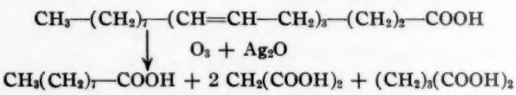


FIG. 4. Degradation of 5:8:11 eicosatrienoic acid.

From this structure, two possible origins suggest themselves: from arachidonic acid by hydrogenation of the terminal double bond or from oleic acid by hydrogenation and chain-lengthening, in a manner similar to the biosynthesis of arachidonic acid. In order to test these alternate hypotheses, fat-deficient rats were injected with carboxy-labeled acetate

TABLE II
Monocarboxylic Acids Resulting from Oxidation of Trienoic Concentrate

Acid	Carbon Atoms	Mole Per Cent	
		Actual	Corrected for Over-oxidation
Decanoic	10	Trace	Trace
Nonanoic	9	82.8	92.7
Octanoic	8	9.0	0
Heptanoic	7	7.5	7.3
Hexanoic	6	0.7	0

treated in the same manner. Second portions of both acids were hydrogenated and degraded stepwise as in previous experiments. That

TABLE III
Dicarboxylic Acids Resulting from Oxidation of Trienoic Concentrate

Acid	Carbon Atoms	Mole Per Cent	
		Actual	Corrected for Over-oxidation
Azelaic	9	5.5	5.5
Suberic	8	4.9	4.9
Pimelic	7	7.1	7.1
Adipic	6	4.0	(4.0)*
Glutaric	5	70.8	81–82.5
Succinic	4	11.7	0–1.5

* Adipic acid was shown by control experiments to be an artifact arising from the polyadipate packing of the gas chromatographic column. Hence it was disregarded in calculating the mole percentage of the other dicarboxylic acids.

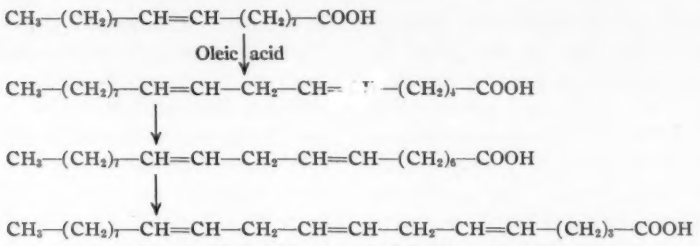
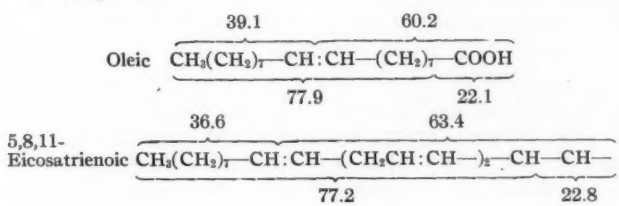


FIG. 5. Transformation of oleic acid. See text.

the oleic acid contained some impurities was indicated by gas-liquid chromatography, which was also used to purify it. From the purified oleic acid were derived, by oxidation, azelaic and pelargonic acids. The trienoic acid fraction was somewhat more complex. Acids derived by oxidation of this fraction are shown in Tables II and III.

From these tables the following conclusions can be drawn: the only monocarboxylic acids formed primarily by the oxidation were pelargonic (C_9, 92.7 per cent) and heptanoic (C_7, 7.3 per cent). Turning to the dicarboxylic acids, we find only 82.5 per cent glutaric acid, which, with pelargonic, was derived from the major 5:8:11-eicosatrienoic acid. Therefore this acid amounted to only 82.5 per cent of the mixture and about 10.2 per cent of the pelargonic acid was derived from some other source. Azelaic acid (C_9, 5.5 per cent), derived from oleic acid, would account for this portion of it. The remaining 4.7 per cent corresponds to the amount of suberic acid (C_8, 4.9 per cent) and indicates a corresponding amount of 8:11-eicosadienoic acid, a possible intermediate in biosynthesis of the eicosatrienoic acid. The amounts of pimelic acid (C_7, 7.1 per cent) corresponds exactly with the amount of heptanoic acid, and would indicate this amount of 7, 10, 13-eicosatrienoic acid. These considerations are shown in Table IV.

The activities of the various degradation products led to the following distribution:



First, it can be seen from the activity of the terminal half of the eicosatrienoic acid that it was derived, not from arachidonic acid but from acetate, since in the former case, it would have contained no activity. Second, from the ratios of activities of the various portions of the eicosatrienoic acid and the corresponding ratios from oleic acid, we can conclude that 5:8:11-eicosatrienoic acid is synthesized from acetate through oleic acid by the fat-deficient rat. This conclusion leads directly to another, namely, that the transformation of oleic acid to 5,8,11-eicosatrienoic acid follows a pathway similar to that of the transformation of linoleic to arachidonic acid. The postulated pathway is shown in Figure 5.

It will be noted, first, that each step in the pathway corresponds to one in the transformation of linoleic acid and, second, that evidence for the occurrence of one of the postulated intermediates, 8,11-eicosadienoic acid, has been presented.

If, in the fat-deficient rat, oleic acid may be

TABLE IV
Composition of Trienoic Concentrate

Acid	Mole Per Cent
5,8,11-Eicosatrienoic	81–82.5
8,11-Eicosadienoic	4.8–5.0
7,10,13-Eicosatrienoic	7.1–7.3
Oleic (9-octadecenoic)	5.4–5.6

transformed into a polyunsaturated acid, a similar transformation may be postulated for palmitoleic acid, which is a major component of the fatty acids of rats in this state.[11] Evidence for such a transformation can be deduced from the probable presence of 7,10,13-eicosatrienoic acid in the mixture. This acid could be derived from palmitoleic acid by steps similar to those postulated above for the other acids.

COMMENTS

From the examples presented it can be seen that the polyunsaturated fatty acids of the animal's body can be formed by successive dehydrogenation and chain-lengthening steps using as starting materials the unsaturated acids obtained through the diet or synthesized from acetate via the saturated acids. The additional double bonds are introduced in the 1:4 relationship to the original double bonds and toward the carboxy group.

$$R{-}CH{=}CH{-}CH_2{-}CH_2{-}CH_2{-}(CH_2)_n{-}COOH$$

$$\downarrow$$

$$R{-}CH{=}CH{-}CH_2{-}CH{=}CH{-}(CH_2)_n{-}COOH$$

If a double bond would logically be introduced into a position $\alpha\,\beta$ or $\beta\,\gamma$ to the carboxy group, apparently two carbons (acetyl coenzyme A) are added to the carboxy end of the molecule and the additional double bond can be introduced in a "safe" position. As substrates for this alteration, use can apparently be made of any unsaturated fatty acid available in which the distance from the existing double bond system to the carboxy group is not too great. At the present time, the following fatty acids have been found to serve: linolenic acid and its products, linoleic acid and its products, oleic and its products and palmitoleic acid and its products. Of these families of acids only the linoleic family has been found to cure deficiency of essential fatty acids. In this case, however, the parent compound may actually be 12-octadecenoic acid, which, by virtue of its low concentration in the dietary or synthetic fat, is generally unavailable as a substrate for further dehydrogenation. Two reports[8,13] have indicated that this acid does not cure the symptoms of deficiency of essential fatty acids. However, a consideration of the reported methods of preparation in both cases leads to the conclusion that it has, in fact, probably not been tested. As products of these alterations can be found, in general, C_{20} acids with three, four and five double bonds and C_{22} acids with five and six double bonds. Some more highly unsaturated acids with greater chain length may be found in the mammalian body, but in very small amounts.

Interpretation of these observations may possibly be made on the basis of interplay of three enzymes.

The first, which could be termed a polydehydrogenase, is concerned with the addition of double bonds toward the carboxy group of unsaturated fatty acids. It may have greatest affinity for C_{18} trienoic acids and for the C_{20} trienoic acid of the linoleic family since these are not found in significant concentrations in animal tissues and, if fed, disappear immediately to give rise to more highly unsaturated products. Next in affinity for this enzyme might be the C_{18} dienes of the linolenic and linoleic families and the C_{20} diene of the oleic family since these acids are found in the tissues and, if fed, can be deposited or used readily for oxidation. A low affinity must exist for the monoenoic acids and affinity for this enzyme must decrease very abruptly with chain lengths above twenty-two carbons.

The second enzyme postulated in these transformations is an acyl transferase which transfers the unsaturated fatty acids (presumably as CoA derivatives) to the methyl group of acetyl CoA, thus accomplishing the chain-lengthening process. Evidence for the existence of such an enzyme has come from many sources: the interconversion of palmitic and stearic acids,[14] the families under present discussion and possibly, the mitochondrial systems observed by several workers[15,16] which would apparently add or subtract acetate but would not synthesize long-chain acids from acetate. It is possible that this enzyme forms part of the normal fatty acid degradation system but that its low affinity for the longer chain acids precludes complete degradation in most cases. Additional evidence for this enzyme in the reverse sense, may be found in the appearance

of C^{14} activity beyond the carboxy group of acids which were fed as the carboxy-labeled molecules.

Finally, it may be postulated that the normal fatty acid degradation system can operate on these unsaturated acids, particularly when they are converted to the $\alpha\beta$ or $\beta\gamma$ unsaturated compounds, which are normal intermediates in the degradation process.

Relative affinities of the various fatty acids for these enzymes could be invoked to explain most of the observations discussed today. For example, the appearance of C_{20} trienes in the animal in the absence of dietary linoleic acid could stem from the fact that with lower concentrations of the preferred substrate, linoleic acid, oleic acid may now compete favorably for the polydehydrogenase and be converted to the acid in question. Likewise, the relative activity of C_1 and C_3 of the C_{20} acids formed from carboxy-labeled linoleic, linolenic and γ-linolenic acids may be a function of the relative affinity of these acids for the polydehydrogenase and degradation systems, the latter removing active acetate which would be used for chain-lengthening.

It is possible, of course, that the ultimate true explanation for the transformations of the polyunsaturated acids will rest on an entirely different basis. However, at present, the proposed system serves to explain existing data on a logical basis and to predict results leading to further experimental verification. Both of these qualities are properties of a useful hypothesis.

SUMMARY

The formation and transformation of the polyunsaturated fatty acids in the animal body have been shown to be part of an orderly system similar to known reactions of saturated and monounsaturated acids. The results have led to a theoretical consideration of the mechanisms involved.

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Unsaturated Fatty Acids and Plasma Lipids

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DIETARY protein has been shown to depress the atherogenic effect of dietary cholesterol and fat in chickens.[1-3] The serum cholesterol, serum lipid and β-lipoprotein levels of birds fed a low protein diet were found to be higher than those fed a high protein diet. Furthermore, it was noted that the energy value of a diet as calculated from the total caloric intake of carbohydrate, fat and protein, had a relationship to serum cholesterol levels. The energy-to-protein (E/P) ratio of a food item for convenience can be calculated by dividing the total available calories in 100 gm. of the food item or diet by its protein content.

An analysis of variance[3] showed that at low or moderate levels of corn oil, statistically significant lower serum and carcass cholesterol levels were noted on a high protein diet as compared to a low protein diet (Table I). When the energy supplied by dietary corn oil was increased from 1.3 to 57.1 per cent, the serum cholesterol value decreased from 200 to 136 mg. per 100 ml. at a low protein level (E/P ratio 22.6) and from 166 to 129 mg. per 100 ml. at a high protein level (E/P ratio 11.5). Thus, dietary corn oil decreased serum cholesterol levels. However, the level of dietary protein influenced the cholesterol-depressing effect of corn oil in the chick, as lower serum cholesterol levels were noted in birds kept on the high protein diet as compared with those on the low protein diet. It was interesting to note that almost twice as much total carcass fat was found at an E/P ratio of 22.6 than at an E/P ratio of 11.5 or approximately 9 and 5 per cent, respectively. Variations in dietary fat or dietary protein did not alter the percentage of total carcass fat significantly as long as the E/P ratio remained constant.

The influence of the E/P ratio on serum and carcass cholesterol levels was independent of the type of dietary fat (Table II). A "hard" fat such as beef tallow gave results similar to a "soft" fat such as corn oil. That is, higher serum cholesterol levels were noted at the lower dietary protein levels whether the diet contained a "hard" or a "soft" fat. A larger percentage of carcass fat and higher carcass and serum cholesterol values were obtained at E/P ratios between 20 to 30 than at E/P ratios of 5 to 10 with either beef tallow or corn oil (Table III). However, dietary fat did have an effect over and above the E/P ratio; this effect was most pronounced at the high E/P ratios.

At comparable E/P ratios, lower serum cholesterol levels were noted in chicks fed a high fat diet as compared to a low fat diet. For example, birds fed 25 per cent beef tallow had lower serum cholesterol levels than those fed 0.2 per cent beef tallow, and those fed 20 per cent corn oil had lower levels than those fed 1 per cent corn oil. Although not strictly comparable because of differences in protein source and fat level, birds receiving 20 per cent corn oil also had lower carcass and serum cholesterol levels than those receiving 25 per cent beef tallow. On the other hand, when flooding of tissue with fat did not occur, i.e., at low fat levels, a high protein level tended to cancel out differences between the effect of "soft" versus

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TABLE I

Effect of Variations in Dietary Protein and Fat on Growth, Protein and Fat Composition and Carcass and Serum Cholesterol Levels of Chicks*

Dietary (per cent)		Energy from Fat (calories) (%)	Average Weight of Birds (gm.)	Carcass Composition (per cent)		Cholesterol Level (mg. per 100 ml.)	
Corn Oil	Protein			Fat	Protein	Carcass	Serum
E/P Ratio 22.6							
0.6	15.1	1.3	358	9.6	17.7	112	200
8.3	16.8	19.9	382	9.1	18.4	98	180
18.1	18.8	38.5	399	8.3	18.1	95	176
30.9	21.2	57.1	371	9.7	17.8	99	136
E/P Ratio 11.5							
0.6	30.0	1.3	405	4.1	18.8	87	166
8.4	33.4	19.6	449	4.7	19.0	80	140
18.2	37.7	37.9	440	5.7	18.6	76	136
31.1	42.3	56.3	434	5.6	18.7	75	129

* In this series 240 one day old male chicks were kept on the same basal diet for seven days and then divided into eight treatments of three replicates of ten birds each. They were kept in standard chick batteries in a randomized block design and fed the eight experimental rations *ad libitum* for a three-week period. Cholesterol values of six individual chicks from each treatment (two from each replicate) were used for an analysis of variance, the figures reported in this table represent an average of six cholesterol values in each treatment.

Least significant difference of serum cholesterol = 26.3 and 28.0 mg. per 100 ml. and 5 and 1 per cent levels, respectively.

Least significant difference of carcass cholesterol = 13.3 mg. per 100 ml. at 5 per cent level.

"hard" fat on serum and carcass cholesterol levels. Chicks on 1.0 per cent corn oil and 0.2 per cent beef tallow had serum cholesterol values of 194 and 233 mg. per 100 ml. at an E/P ratio of 17 to 18, and 145 and 144 mg. per 100 ml. at an E/P ratio of 7 to 8, respectively.

The percentage of total carcass fat did not seem to be influenced by the type of dietary fat. In every case the percentage of total carcass fat seemed to be directly dependent on the E/P ratio and independent of the level or type of dietary fat. These results also seemed to indicate that the capacity to store cholesterol was significantly higher when the body tissues contained a larger percentage of fat.

In another series of experiments on "hard" and "soft" fat,[4] one day old chicks were kept for four weeks on diets which contained 10 per cent lard or corn oil and from 50 to 6 per cent protein (Table IV). Statistically higher serum cholesterol values were noted in birds which had received lard than those on corn oil and 25 or

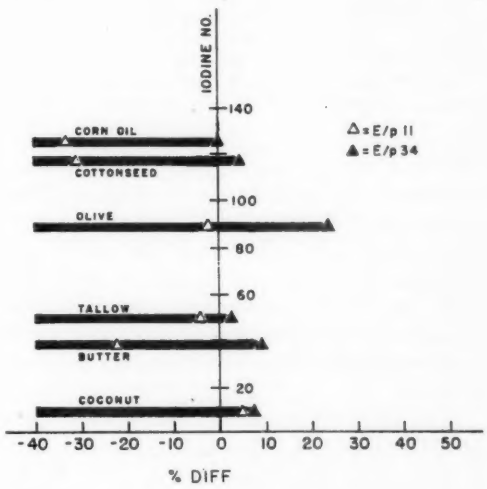

FIG. 1. The relationship of the iodine value of various fats to the serum cholesterol level in chicks fed low (E/P 34) and high (E/P 11) protein diets with deviations in mg. per 100 ml. from chicks fed corn oil at an E/P ratio of 34.

TABLE II

Effect of Energy/Protein Ratio on Percentage of Carcass Fat and Carcass and Serum Cholesterol Levels*

Dietary Protein (%)	E/P Ratio	Carcass Fat (%)	Cholesterol (mg. per 100 ml.)	
			Carcass	Serum
0.2 Per Cent Beef Tallow				
15	23	13.1	98	237
20	17	10.5	84	233
35	10	6.5	65	200
45	8	4.7	59	144
25 Per Cent Beef Tallow				
15	30	15.7	123	208
20	22	13.5	125	200
35	13	8.9	111	186
45	10	6.9	110	160

* In this series 600 one day old male chicks were divided into twenty treatments with four levels of fat (0.2, 10, 20 and 25 per cent) and five levels of protein (15, 20, 25, 35 and 45 per cent). The twenty experimental diets were fed *ad libitum* for a four-week period. The figures reported in this table represent an average of six cholesterol values for eight treatments, that is, only the two extreme levels of fat at each of the four protein levels. The linear regression coefficient for serum cholesterol on the basis of all twenty treatments = −2.0 mg. per cent when 1 per cent carbohydrate was replaced by 1 per cent protein.

Average body weight at 0.2 per cent tallow, 331 ± 64 gm.; at 25 per cent tallow 272 ± 45 gm.

TABLE III

Effect of Energy/Protein Ratio on Percentage of Carcass Fat and Carcass and Serum Cholesterol Levels*

Dietary Protein (%)	E/P Ratio	Carcass Fat (%)	Cholesterol (mg. per 100 ml.)	
			Carcass	Serum
1 Per Cent Corn Oil				
20	18	10.8	71	194
30	12	7.1	78	167
40	9	4.8	63	161
50	7	3.0	56	145
20 Per Cent Corn Oil				
20	23	13.9	79	159
30	15	8.6	77	119
40	12	6.2	40	114
50	9	5.0	44	105

* In this series 240 one day old female chicks were divided into eight treatments of three replicates of ten birds each and fed the experimental diets *ad libitum* for a three-week period. The figures reported in this table represent an average of six cholesterol values in each treatment. The linear regression coefficient = −2.2 mg. per 100 ml. when 1 per cent carbohydrate was replaced with 1 per cent protein.

Average body weight at 1 per cent corn oil, 239 ± 2.6 gm.; at 20 per cent corn oil, 241 ± 6.4 gm.

12.5 per cent protein. Thus, in comparison with lard, corn oil did have an effect over and above the E/P ratio at a particular protein level. However, this effect was most pronounced on a low protein diet of high E/P ratio; on a high protein diet of low E/P ratio, a numerical but not a statistical difference in serum cholesterol was noted between lard and corn oil.

The effect on serum cholesterol of various edible oils and fats at low and high dietary protein levels is represented in Figure 1. In this figure the serum cholesterol level of chicks fed corn oil and a low protein diet (E/P ratio, 34) has been used as a point of reference.[5–7] The results indicated that the substitution of olive oil, tallow, butterfat or coconut oil for corn oil increased serum cholesterol values in chicks. At a higher protein level (E/P ratio, 11) all of the fats except coconut oil gave lower serum cholesterol values than corn oil at an E/P ratio of 34. Thus, for chicks, the serum cholesterol value is dependent on the dietary protein level as well as the degree of unsaturation of the dietary fat.

Mattil[8] recently stated that the linoleic acid content of the visible fat in our diet has increased from 6 per cent in 1880 to 17 per cent in 1956 (Table V). In order to determine whether a shift in the consumption of a mixture of edible fats can shift serum cholesterol values in chicks, a mixture of fats was prepared from the per capita consumption of visible fats in 1921 as compared with 1956 (Table VI). These data and methods of procedure suffer from two defects: one, whether or not the per capita consumption of fat as listed[9] is accurate;

TABLE IV

The Effect of the E/P Ratio and the Type of Dietary Fat on Weight Gain and Serum Cholesterol Level in Chickens

Dietary Protein (%)	E/P Ratio	10 Per Cent Dietary Fat				
		Lard		Corn Oil		
		Average Weight Gain (%)	Serum Cholesterol (mg. per 100 ml.)	Average Weight Gain (per cent)	Serum Cholesterol (mg. per 100 ml.)	Difference (mg. per 100 ml.)
50.0	8	415	119	430	95	+24
25.0	16†	450	178	474	117	+61
12.5	32†	256	268	281	194	+74
9.0	45†	170	271	172	194	+77
6.0*	67†	47	222	63	206	+16

* Chicks fed a diet containing 6 per cent protein showed extremely poor growth. Four out of sixteen birds were lost during the experiment because of protein deficiency.

† Difference in serum cholesterol levels between lard and corn oil treatments significant at 5 per cent level.

Linear regression coefficient for serum cholesterol: Lard = +1.74, mg. per 100 ml. when E/P ratio increased by one; corn oil = +1.94, mg. per 100 ml. with E/P ratio increased by one.

and two, whether or not the composition of the fats themselves have changed. The latter may not be true for lard and butterfat, but the composition of the shortenings and margarine offered for sale in 1956 are not similar to those on sale in 1921.

A simulated mixture of butterfat, lard, shortening and margarine bought as stock items in a supermarket and formulated as listed for the per capita consumption for 1921 and for 1956 was fed to chicks at a 10 per cent level in a low and high protein diet. The results indicated that on a low protein diet (E/P ratio of 34) both mixtures increased the serum cholesterol values when compared with corn oil (Fig. 2). However, on a high protein diet at an

TABLE V

Total Percentage of Linoleic and Saturated Fatty Acids Consumed in Visible Fats[8]

Year	Linoleic Acid (%)	Saturated Acids (%)
1880	6	49
1910	17	43
1920	13	45
1956	17	33

TABLE VI

Per Capita Consumption of Fats[9] (in pounds)

Fat	1921	1956
Butter	16.0	8.7
Lard	10.6	9.9
Margarine	2.0	8.2
Shortening	6.9	10.9
Edible oils	3.5	10.0

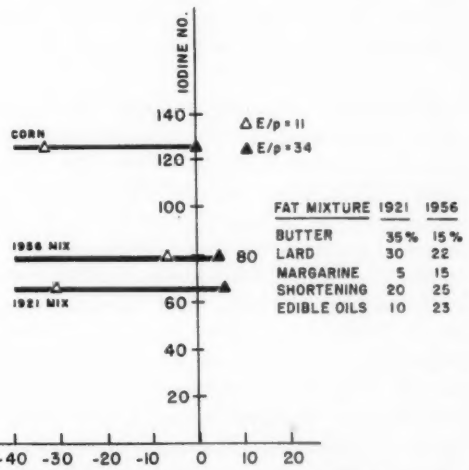

FIG. 2. The relationship of dietary protein level to the serum cholesterol level in mg. per 100 ml. in chicks fed two composite fat mixtures as compared with chicks fed corn oil.

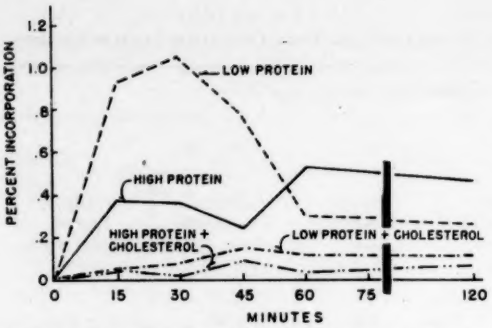

FIG. 3. The percentage of incorporation of acetate-1-C^{14} into liver cholesterol in chicks which had been kept on low and high protein diets.

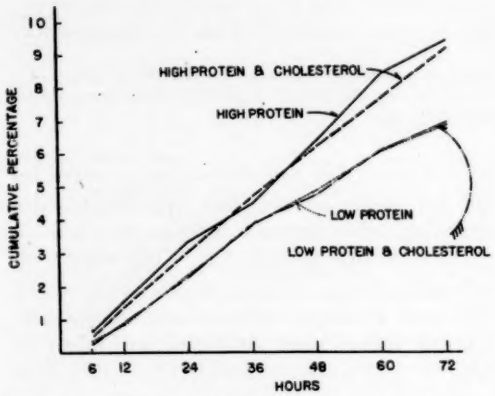

FIG. 4. The cumulative percentage excretion of cholesterol-4-C^{14} as bile acids obtained from chicks kept on low and high protein diets.

E/P ratio of 11, the 1921 mixture gave results which were closer to corn oil than the 1956 mixture in spite of the higher iodine value of the latter. Thus, there may be other factors in addition to the dietary protein level and the degree of unsaturation in the dietary fat which influence serum cholesterol levels in the chick.

The manner in which dietary protein lowers serum cholesterol levels in chicks is not known. Preliminary results in our laboratory indicate that the anabolism as well as the catabolism of cholesterol is influenced by the dietary protein level. A higher initial incorporation of acetate-1-C^{14} into cholesterol occurred in the livers of birds which had been kept on the low protein diets as compared with those on the high protein diets (Fig. 3). The presence of dietary cholesterol stimulated the incorporation of more acetate-1-C^{14} into liver cholesterol in chicks which had been kept on the low protein diet as compared with those on the high protein diet. However, dietary cholesterol significantly depressed the rate of incorporation of acetate-1-C^{14} into cholesterol. Although dietary cholesterol depressed cholesterol biosynthesis, it stimulated the rate of incorporation of acetate-1-C^{14} into fatty acids. The ratio of incorporation of acetate-1-C^{14} into fatty acids was larger in the presence of dietary cholesterol than in the absence of cholesterol.

The results of studies of cholesterol catabolism indicated that more cholesterol-4-C^{14} was excreted by birds which had received the high protein diet as compared with those on the low protein diet during a seventy-two-hour test period (Fig. 4). The presence of dietary cholesterol did not seem to affect the rate of cholesterol-4-C^{14} excretion. Chicks in which the bile duct had been cannulated excreted only 8 per cent of the cholesterol-4-C^{14} as bile acids, while rats excreted 52 per cent of cholesterol-4-C^{14} as bile acids during the same period of time. Furthermore, the increased flow of bile due to the cannulation itself was a factor in cholesterol metabolism.

Cannulation of the bile duct caused a significant decrease in serum cholesterol and serum lipoprotein levels, while ligation of the bile duct caused a significant increase. In the latter case the lipoprotein pattern became more heterogeneous with a significant increase of the lipoprotein in the low S_f range. These results indicated a close relationship between bile flow and serum cholesterol and lipoprotein levels.

It is thus apparent that both the anabolism and catabolism of cholesterol are influenced by nutritional as well as physiologic factors. However, how these factors influence the development of atherosclerosis is still to be determined.

Dam and co-workers[10,11] noticed an increase in the cholesterol content of the aorta of the chick when fat and cholesterol had been included in the dietary regimen. This increase occurred whether the fat was unhydrogenated or hydrogenated. However, as Geer et al.[12] have pointed out, an analysis for cholesterol

TABLE VII
Lipid Content of Human Aorta

	Extractable Lipid (%)
Adventitia	9.5
Media	2.9
Intima with lesion	4.9
Intima without lesion	1.3

content of the entire aorta may not reflect differences in the cholesterol content of the involved and non-involved areas. We have noted differences in the total lipid content of the adventitia, media and intima layer of a human aorta (Table VII). Furthermore, the involved area contained more extractable lipid than the non-involved area.

CONCLUSIONS

It is evident dietary as well as physiologic factors influence the serum cholesterol level. Although the essential unsaturated fatty acids may be involved in cholesterol transport, it does not necessarily follow that an excess of dietary linoleic acid is desirable. The proportion of dietary carbohydrate, fat and protein over a protracted period of time may influence the degree of formation of plaques and atherosclerosis more dominantly than "soft" fats. It seems that the arterial wall *per se* should receive as much study as the serum cholesterol value.

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The Antihypercholesteremic Effect of Essential Fatty Acids in Hypercholesteremic Dogs

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HANSEN and Wiese[1] demonstrated the dog's need for dietary unsaturated fats. These same investigators[2] showed serum cholesterol values were lower in dogs fed unsaturated fatty acids than in dogs fed butter. Tsai et al.[3] reported later serum cholesterol values were lower in dogs fed vegetable oils.

The availability of naturally hypercholesteremic dogs afforded us an opportunity to study, under natural dietary conditions, the antihypercholesteremic effects of unsaturated fatty acids and of vegetable oils.

MATERIALS AND METHODS

Naturally hypercholesteremic dogs were obtained by screening our stock colony for dogs which had abnormal eye grounds and hypercholesteremia. The dogs were housed individually in metabolic cages, with water constantly available. They were fed twice a day, once about 11:00 A.M. and again about 4:30 P.M. In addition to one can of prepared dog food, they were given mineral-enriched whole milk supplements as indicated in the figures. Food and milk supplements were supplied on a per kilo basis and were adequate to maintain constant weights of the dogs. Plasma cholesterol levels (method of Anderson and Keys[4]) were determined weekly on each dog throughout each test period. Test materials were administered to dogs in capsule form. The control dogs received an equivalent number of empty capsules. Three experiments were performed in which soybean oil, linoleic acid or ethyl linoleate were tested for their antihypercholesteremic effects. The experimental details of each test are presented with the results.

Linoleic acid was prepared in our laboratory from safflower seed oil by the method of Swern and Parker.[5] Ethyl linoleate was prepared from linoleic acid using acid catalyzed esterification. The soybean oil emulsion was prepared to contain 60 per cent fat in an aqueous medium. The analytical data obtained on the fatty acids and fat used in these experiments are presented in Table I.

RESULTS

Experiment 1

The antihypercholesteremic effect of orally administered linoleic acid was compared with an equivalent amount of unsaturated fatty acids in soybean oil in naturally hypercholesteremic dogs (Fig. 1). Neither substance, administered orally once daily with the morning meal at a dosage of 200 mg./kg. body weight/day, had antihypercholesteremic effects in these dogs during a one-week period. When the levels of linoleic acid or equivalent unsaturated fatty acids administered as soybean oil were doubled to 400 mg./kg./day once daily, a significant decrease in plasma cholesterol was observed in the dogs receiving linoleic acid (group 2) but not in those fed soybean oil (group 3). When the unsaturated fatty acid dosage was reduced to 200 mg./kg./day during the last two weeks of the experiment, the plasma cholesterol values tended to remain

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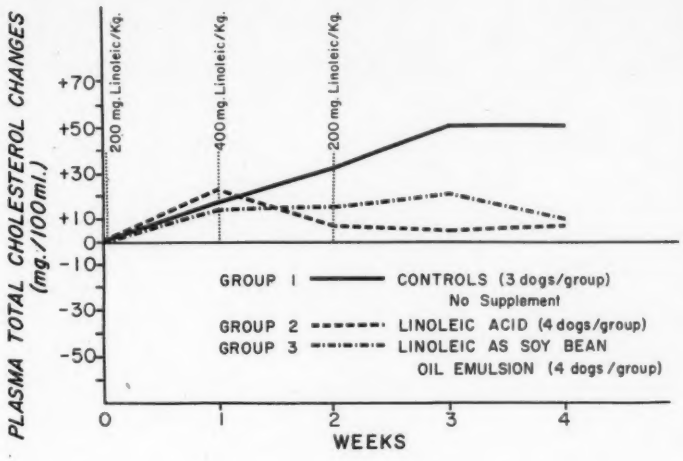

FIG. 1. Antihypercholesteremic effect of orally administered linoleic acid or its equivalent as soybean oil emulsion in dogs. Starting average plasma total cholesterol value 162 mg. per 100 ml.

TABLE I

Composition of Unsaturated Fatty Acids, Fatty Acid Esters and Soybean Oil Fed to Dogs

Material	Purity* (%)	Iodine Value		Peroxide Value‡
		Theory	Found†	
Linoleic acid§,¶				
PJC-3-253-A	95.5	181	178	0.0
Ethyl linoleate**				
PJC-3-289-B	94.6	164.5	163.0	0.0
PJC-4-20-A	96.9	163.5	163.5	
Soybean oil (Lot No. 587)				
Oleic acid	24.8		127	0.95
Linoleic acid	47.2			
Linolenic acid	7.12			
Saturated fatty acids	16.6			
Free fatty acids††	<0.1			

* Method Cd-7-48, Official and Tentative Methods of American Oil Chemists Society.

† Method Cd-1-25, Official and Tentative Methods of American Oil Chemists Society.

‡ Method Cd-8-53, Official and Tentative Methods of American Oil Chemists Society.

§ Linoleic acid was isolated from safflower oil according to the method of Swern and Parker.[8] It was purified finally by molecular distillation through a 2-inch, Rota-film molecular still. Digitalin precipitable material <0.005 per cent.

¶ Infrared study identified this material as natural cis,cis-octadecadien-9,12-oic acid.

** Ethyl linoleate was prepared by *p*-toluenesulfonic acid catalyzed esterification of linoleic acid and was purified finally by one molecular distillation.

†† Method Ca-5-40, Official and Tentative Methods American Oil Chemists Society.

at a lower level than was observed in the control dogs.

Experiment 2

When linoleic acid was administered orally to dogs at a level of 400 mg./kg./day twice a day at feeding time in the morning and again in the afternoon, a steady drop of plasma total cholesterol occurred during the four-week test period. This downward trend continued throughout the eleven-week observation period (Fig. 2). The total drop in plasma cholesterol values for the dogs treated with linoleic acid (group 5) averaged −69 mg. per 100 ml. (−81 and −57 mg. per 100 ml.), while the control dogs (group 4) averaged +7 mg. per 100 ml. (+56, −5 and −29 mg. per 100 ml.).

Experiment 3

This experiment tested the ability of the ethyl ester of linoleic acid to reduce plasma cholesterol. Ethyl linoleate, administered orally at a level of 200 mg./kg./day twice daily to experimental dogs, caused a consistent reduction of plasma total cholesterol (Fig. 3). The plasma cholesterol-lowering effect of ethyl linoleate was evident even when whole milk was added to the diet. The downward trend in plasma cholesterol levels continued in the dogs treated with ethyl linoleate for at least four weeks after the supplementation of

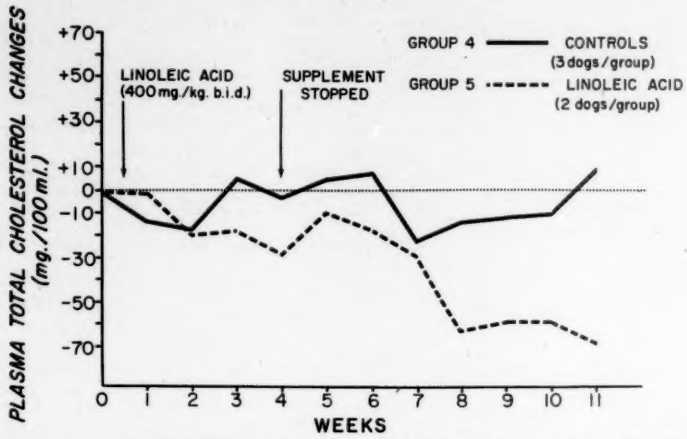

FIG. 2. Antihypercholesteremic effect of linoleic acid when administered orally at a level of 400 mg./kg. twice a day to dogs. Starting average plasma total cholesterol value 190 mg./100 ml.

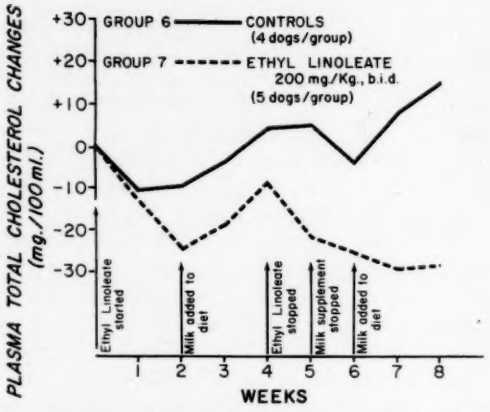

FIG. 3. Effect of ethyl linoleate on plasma total cholesterols in dogs when administered orally at a level of 200 mg./kg. twice a day. Starting average plasma total cholesterol value 186 mg./100 ml.

the ester had been stopped. These persistent postadministration antihypercholesteremic effects of ethyl linoleate were similar to those seen in experiment 2 when linoleic acid was tested.

COMMENTS

The cholesterol values of the naturally hypercholesteremic dogs used in this study may be considered to be moderately elevated or high, if one considers cholesterol levels in dogs on controlled diets (80 to 120 mg. per 100 ml.)[6] as representing normal plasma cholesterol values for dogs. Deuel[7] points out that essential fatty acids are required for the normal distribution and metabolism of cholesterol. Bloor[8] noted earlier that the unsaturated fatty acids were important for the transportation and metabolism of cholesterol. In the present study both linoleic acid and ethyl linoleate, essentially free of sterols, were effective agents for reducing plasma total cholesterol; thus the identity of essential fatty acids as an active antihypercholesteremic component of vegetable oil seems established. Our observations are in accord with the findings of others who have shown that unsaturated fatty acids have beneficial effects on cholesterol metabolism.[9-15]

SUMMARY

Naturally hypercholesteremic dogs serve as useful animals in determining the value of unsaturated fatty acids for reducing plasma total cholesterol. Unsaturated fatty acids (linoleic or ethyl linoleate) bring about a significant and consistent reduction in plasma cholesterol.

ACKNOWLEDGMENT

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Speculations Concerning the Functions of Serum Cholesterol

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"Lernen wir traümen, dann finden wir vielleicht die Wahrheit."
KEKULÉ

No lipid parameter is measured more frequently or discussed as endlessly as is serum cholesterol. However, the possible functions of serum cholesterol are seldom touched upon. I would like to indulge in a few speculations in this area.

Serum cholesterol is present in both the free and esterified form, and the ratio between the free and esterified cholesterol is constant with the exception of certain liver diseases and lipidoses.[1] In dogs, an equilibrium between the whole blood free cholesterol and the plasma ester cholesterol has been demonstrated.[2] Isotope experiments have shown, however, that free and ester cholesterol do not appear in the serum at the same rate. In human subjects who have been fed radioactive cholesterol[3] the peak serum radioactivity occurs two to three days after feeding, with more of the radioactivity present initially in the free cholesterol. In human subjects biosynthesizing cholesterol from labeled acetate[4] the peak serum radioactivity is seen within eight hours, but the initial difference between specific activities of the free and ester cholesterol still holds. Eventually the specific activity of the ester cholesterol becomes higher than that of the free cholesterol, and remains so. The inability of hepatectomized rats[5] to replace lost cholesterol ester and the absence of cholesterol-C^{14} in the plasma sterol ester[6] of hepatectomized dogs fed acetate-C^{14} demonstrates that the liver is responsible for the appearance of cholesterol ester. The observation of Portman and Sinisterra[7] may be relevant to the following discussion. They found that in monkeys given cholesterol-C^{14} intravenously, the biological half-life of serum cholesterol was 8.8 days, while orally administered labeled cholesterol showed a die-away curve of serum cholesterol with two distinct components exhibiting half-life of 2 to 6.6 days and 17 to 34 days, respectively.

Assume that the free and ester cholesterol in the serum had distinct and separate functions. I do not mean to imply two distinct and unrelated pools, but rather that when cholesterol is present in the serum in the free state it is available to perform certain metabolic functions, and when it is present as esterified cholesterol it may be available to perform others. Any given cholesterol molecule, according to this supposition, may go from the free form to the ester form. In doing so the specific metabolic functions change. What could be assigned as the metabolic role of the free cholesterol?

FREE CHOLESTEROL

First, there is the rapid exchange with the red blood cell cholesterol,[8] an exchange not noted with ester cholesterol. Thus, only serum free cholesterol is required for maintenance of the integrity of the red blood cell structure. The constancy of the red blood cell cholesterol

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content has been demonstrated in man over a wide range of clinical conditions.[8] Eightfold elevations of the serum cholesterol resulted in no increase in the red blood cell cholesterol[9] in rabbits fed large doses of cholesterol.

Cholesterol is a precursor of the bile acids, adrenocortical hormones and sex hormones.[11] Landon and Greenberg[12] have suggested that the circulating cholesterol is the precursor of the cholesterol found in the testes, kidneys, adrenals, spleen and lungs. Assume this cholesterol, the hormonal precursor, is derived from the plasma free cholesterol. LeRoy[13] has estimated that 0.5 to 1 per cent of the plasma free cholesterol which is utilized daily is transformed to corticosteroids. Estimates of cholesterol conversion to bile acids are also available,[14,15] but what portion of this comes from the circulating cholesterol is not known.

Some of the carbon atoms biologically removed from the cholesterol molecule may be used for synthesis of fatty acids[16,17] and glycogen[17] as has been shown in experiments using randomly labeled sterol. Assume that these, too, are contributions of the free cholesterol.

Another important function of the circulating cholesterol may be in the "replacement" or turnover of tissue cholesterol. Chevallier[18,19] estimates that almost half of the tissue cholesterol of the rat is "replaceable," the amount being turned over varying from zero for the brain cholesterol to almost 100 per cent for tissues such as the liver, adrenals and intestines. Suppose, then, that the major function of the free cholesterol is that of an integral metabolite, serving as a substrate for steroid synthesis and for the renewal of red blood cells and tissues.

ESTER CHOLESTEROL

On the basis of his own esterification studies Favarger[20] concluded that the ester cholesterol was a means for fatty acid transport. Popjak[21] fed cholesterol to rabbits on a fat-free diet and showed a progressive increase of all plasma lipids and a concomitant depletion of body fat depots. The ester:free cholesterol ratio in the plasma rose as feeding continued, the major, if not only, source of fatty acid being body pools. He therefore inferred a role for cholesterol in fat mobilization.

The type of fatty acid which is esterified with cholesterol is also significant. Popjak[21] found that as cholesterol feeding progressed there was a progressive drop in the iodine number of the phospholipid fatty acid and an increase in the non-phospholipid fatty acid. Serum cholesterol is preferentially esterified with unsaturated fatty acids[22,23] and *in vitro* enzymic esterification of cholesterol proceeds more rapidly with unsaturated fatty acids than with saturated fatty acids.[24] It has also been shown that the iodine number of total serum fatty acids of rats is correlated with the unsaturation of the dietary fat.[25] A similar correlation has been observed with respect to the iodine number of the fatty acids of the combined cholesterol ester and neutral fat fractions in dogs.[26]

In man it has been shown that the iodine number of the cholesterol ester fatty acid is a function of the unsaturation of the dietary fat.[27] The cholesterol ester fatty acids are generally derived from the dietary fat and are the most unsaturated in the serum.

Studies on the synthesis of fatty acids in man indicate that practically no linoleic acid is synthesized,[28] yet most of the fatty acids of the sterol ester are unsaturated. Feeding of labeled fatty acids (as their methyl esters) to rats shows a preferentially rapid incorporation of linoleate (over oleate and stearate) into the sterol esters.[29] In their thorough review of the transport of fatty acids, Fredrickson and Gordon[30] conclude that there is no evidence that sterol esters play a significant role in the net transport of fatty acids, but they do not exclude the possibility that cholesterol ester may be a means of transport within the serum. However, Miller, Roheim and Spitzer[31] have shown that cholesterol ester may be a means of transport of fatty acid to the heart.

Bates[32] has found that the half-life of plasma cholesterol ester fatty acids in the dog (7.8 ± 3.4 hours) is much shorter than the half-life of plasma cholesterol itself in either the rat or monkey, and she suggests that one plasma cholesterol molecule might be esterified suc-

cessively with a series of fatty acid molecules while in the plasma. Seven hours has been found to be the half-time for the whole blood free cholesterol–plasma ester cholesterol system in the dog,[2] suggesting a renewal of the entire sterol ester molecule. The serum sterol ester originates in the liver. Louédec has reported that the liver cholesterol esters are not transferred to the serum as such;[33] thus the liver may be the site of transesterification between sterol ester and exogenous neutral fat, as well as the site of cholesterol ester synthesis by other mechanisms. The sterol ester then may function as one of several transport mechanisms for fatty acids and perhaps is the mechanism of choice for the transport of highly unsaturated or essential fatty acids. Kinsell and his associates[27] have speculated that the cholesterol ester functions as a donor of essential fatty acid for phospholipid synthesis.

Both dietary polyunsaturated fatty acids[34] and cholesterol[17] pass through the placenta. This may be used as another piece of evidence for the transport function of cholesterol esters. This may be one source of the incompletely explained hypercholesteremia of pregnancy.

LIPOPROTEINS

One other possible role of serum cholesterol, free and ester, is as an integral structural unit of lipoproteins. One explanation of the fact that cholesterol is distributed in all the lipoprotein fractions is that its function may be structural. The lipoproteins contain most of the other lipid entities, however, and their delicately balanced physical state in the blood probably demands a specific structural make-up for stability. Lindgren et al.[35] have suggested a series of structures for lipoproteins, taking into consideration their size and lipid content. The absolute cholesterol content of various lipoproteins has been studied during fat and cholesterol absorption;[36] only the cholesterol content of the chylomicron-containing fraction is elevated. In man the specific activity of the S_f 3–7, S_f 10–13, S_f 17 and S_f 20+ lipoprotein cholesterol is the same as that of the total serum cholesterol twenty-four hours after administration of cholesterol-H^3,[3] suggesting rapid equilibration of cholesterol between lipoprotein classes.

It is hard to imagine any one segment of the lipoprotein being an inert building block. The dietary alteration of the cholesterol content of various lipoproteins, even in the face of unchanged serum cholesterol levels,[37] also argues against this point. It does suggest the intimate inter-relationship between serum lipids, as affected by diet, and structure and composition of lipoprotein. The rather constant free:ester cholesterol ratio also points towards an attempt at maintenance of physical integrity, but again this may also be a physiological mechanism for keeping cholesterol in solution until it reaches its site of utilization.

I have speculated that the free cholesterol of the serum plays an integral metabolic role, whereas the ester cholesterol is partly involved in fatty acid transport, although it must be recognized that an equilibrium between these two types of cholesterol exists. The real question is how to go about proving some of these hypotheses. The work on free cholesterol might be aided by double-labeling experiments involving carbon- and hydrogen-labeled cholesterol. The labeling of body depots and the observation of their exchange with newly synthesized or ingested sterol may be an answer. The most obvious immediate problem would be in the detection of small amounts of radioactivity. Chevallier[13] has carried out some experiments of this type.

Cholesterol ester as a means of fatty acid transport depends on the availability of labeled unsaturated fatty acids. Carboxyl-labeled oleic, linoleic and linolenic acids have been synthesized by Drs. Howton and Nevenzel,[38–40] but are generally hard to obtain. I should like to suggest a biosynthetic source of labeled unsaturated fatty acids—the chicken egg. The normal egg yolk consists of the following fatty acids: Oleic acid (50%), palmitic acid (27%), linoleic acid (11%), stearic acid (6%), palmitoleic and linolenic acids (6%). It has been shown that the chicken egg is a good source of fat which can be labeled biosynthetically so as to attain fairly respectable levels of specific activity.[42,43] Since the actual composition of the fatty acids in the egg yolk

can be altered by variations in the diet,[44,45] a combination of specific diet plus administration of labeled precursor should yield labeled fatty acids of the desired kind. Obviously, the fed fats cannot be labeled, but the amount of any desired fatty acid can be raised so as to insure good recovery. With enough radioactive precursor, fairly high specific activity should still be attainable. Double-label experiments involving cholesterol and labeled fatty acids may help to resolve some of these problems.

SUMMARY

Speculations concerning the metabolic functions of the free and ester cholesterol of the serum are presented. The discussion is based on the supposition that serum free cholesterol serves a structural purpose and as a precursor of other steroids whereas serum ester cholesterol is part of the fatty acid transport mechanism. The literature bearing on these points is reviewed.

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Thyroid: Fundamental Considerations

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THE STUDY of normal and abnormal physiology of the thyroid has advanced more rapidly in the past ten years than at any time previously. This has been largely due to the availability of newer analytical technics employing radioactive iodine, paper chromatography and zone electrophoresis. At the same time, some progress in the diagnosis and treatment of thyroid disorders has been made to a large extent based on this new knowledge acquired in the laboratory.

I would like to discuss some of the newer developments in "thyroidology," some of which are rather fundamental in scope while others are of more direct clinical interest.

In order to integrate these remarks, lest they seem unrelated and disconnected, I should like to begin with the hypothalamus and pituitary, and continue with the thyroid gland and blood, and the cells of the peripheral tissues.

HYPOTHALAMUS AND PITUITARY

The importance of the hypothalamus in regulation of the thyroid has been recognized clinically for many years. In this connection the occurrence of severe stress preceding the onset of hyperthyroidism is noted rather frequently. For example, development of this disease is observed following termination of pregnancy, after non-specific febrile illness or severe emotional shock. Illustrative of the latter is a case in which a sixteen year old girl, seen several years ago, was irretrievably involved with a young man. Upon learning that he was being inducted into the army, virtually all the manifestations of fairly severe exophthalmic goiter developed in the course of two months. This girl, figuratively speaking, was "out in the cold" so far as her life was concerned. It is tempting to draw comparisons with the experimental animal at this point. It is well known that exposure of the non-hibernating mammal, such as the rat, to cold environmental temperatures of 0 to −4°C. for two weeks is a standard method of producing hyperthyroidism in this species. In either case the role of the hypothalamus cannot be overemphasized.

Further evidence of hypothalamic-hypophyseal influence on the thyroid is available from clinical investigation. There is a well recognized tendency for hyperthyroidism to be familial. This may be in part a function of inherited personality patterns and similar environmental stress to which members of the same family are exposed. Additional explanations may exist and should be looked for. A promising start in this direction has been made by Ingbar et al.,[1] who investigated possible abnormalities of iodine metabolism in euthyroid relatives of patients with Grave's disease. He determined radio-thyroxine turnover rate in twelve clinically euthyroid relatives of five thyrotoxic patients and found it to be significantly elevated in all. Measurement of thyroidal radioiodine uptake in this group of twelve subjects revealed values within the hyperthyroid range, although basal metabolic rates and serum protein bound iodine values were normal. In an additional group of forty-four euthyroid relatives of nineteen patients with Grave's disease, thyroidal radioiodine uptake was found to be increased over the normal. In contrast to what may be achieved in patients with active Grave's disease, a suppression of increased uptakes was produced by the ingestion of 2 to 4 grains of desiccated thyroid daily. These interesting findings strongly suggest the participation of the hypothalamus and pituitary in this familial phenomenon of increased thyroidal avidity for iodine. The reasons for the aug-

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mented rate of peripheral degradation of thyroxine in these eumetabolic asymptomatic relatives are not understood. The significance of this work is that it will contribute a physiologic basis for our ultimate understanding of the etiology and pathogenesis of hyperthyroidism.

Another important development bearing on the role of the hypothalamus in thyroid physiology was described recently by Blanquet and his associates.[2] They found that it was possible to radiate localized sections of the hypothalamus in rats by means of a focused beam of high energy particles from the cyclotron. After radiation of the median eminence of the hypothalamus, certain studies of thyroid function were carried out. It was found that uptake of I^{131} determined *in vivo* was normal or slightly elevated. However, the amino acid content of the thyroid gland was found to be greatly altered. The concentrations of monoiodotyrosine and diiodotyrosine, precursors of the hormone, were normal, but concentration of thyroxine was low. This finding indicates that it is possible to obtain selective effects on thyroid metabolism even in the absence of pituitary lesions, and seems to suggest a control by the median eminence area of the hypothalamus over synthesis of thyroxine.

If there is a clinical example of this type of lesion it has not been recognized up to this time. Cases of congenital goiter have been reported in children in whom I^{131} uptake is generally elevated and the circulating iodine is chiefly in the form of monoiodotyrosine and diiodotyrosine to the exclusion of thyroxine. It is tempting to speculate that perhaps some of these patients represent a previously unrecognized syndrome, hypothalamic goiter, in which the primary lesion is in the hypothalamus. The presence of a goiter would certainly suggest that the fault does not lie with deficiency of pituitary thyroid stimulating hormone.

THYROID

Synthesis of thyroxine by enzyme systems within the thyroid gland is clearly a sequential process. The essential steps may be enumerated in outline as follows: (1) iodide is concentrated by the acinar cells from plasma perfusing the gland; (2) oxidation of iodide to iodine is a prerequisite to the incorporation of the halogen into tyrosine as monoiodotyrosine and diiodotyrosine; (3) coupling of two molecules of diiodotyrosine yields tetraiodothyronine or thyroxine. This sequence represents the major pathway of thyroid hormone synthesis. The presence of small quantities of triiodothyronine in the thyroid gland presumes an additional mechanism to account for its occurrence, but it is uncertain whether thyroidal triiodothyronine arises from coupling of monoiodotyrosine and diiodotyrosine or by deiodination of thyroxine. In either event we are interested in the evolution of thyroxine and in the concept of genetic control of specific enzyme systems involved. It is now apparent that a genetic defect may be manifest in terms of failure of a specific enzyme in the sequence involved in thyroxine manufacture. Thus, it is believed that certain cases of congenital goiter represent isolated genetic defects carrying imperfect or absent enzyme systems normally required in the synthesis of thyroxine.

Considerable interest in the problem of lymphocytic thyroiditis has arisen in the past two years. This has been due to what appears to be a real increase in the frequency of this disease. Skillern et al.[3] have described abnormalities in the serum proteins of patients with Hashimoto's disease, namely high values for gamma globulins and positive flocculation tests. The known association of gamma globulins with circulating antibodies and the infiltration of the gland with plasma cells and lymphocytes suggest that the disease might be explained if these patients were immunized against an antigen in the thyroid gland. Using the technic of diffusion-precipitation reactions in agar gel, it has been demonstrated that the serums of patients with Hashimoto's disease contain autoantibodies against thyroglobulin.[4] The antibody is organ-specific and does not cross react with extracts of the thyroid gland from any of six mammalian species. The level is highest in untreated patients and becomes weaker or negative after thyroidectomy. The demonstration of circulating antibodies against the patient's own thyroglobulin suggests a new approach to the

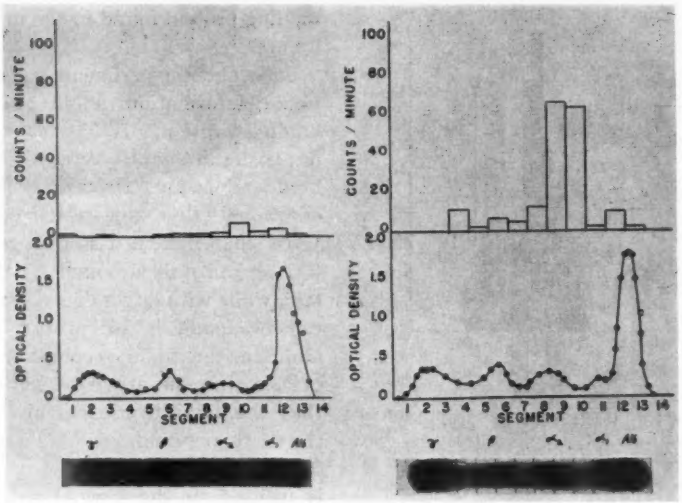

FIG. 1. Localization of protein-bound I^{131} by paper electrophoresis in an euthyroid (A) and a hyperthyroid (B) patient eight and two days, respectively after I^{131} administration. The bottom strips are photographs of the stained paper strips. The middle graphs represent the protein concentration of the strips (dye elution method). The top graphs show the relative radioactivity of the segments. From: DEISS, W. P., ALBRIGHT, E. C. and LARSON, F. C. *J. Clin. Invest.*, 31: 1000, 1952.[5]

etiology of lymphocytic thyroiditis. It may be supposed that limited extravasation of thyroglobulin stimulates invasion by lymphoid tissue with attendant local antibody production. Sufficient antibody may be produced to damage adjacent follicles and release more thyroglobulin which in turn elicits greater antibody production. The progressive interaction of autoantibody and thyroglobulin in the gland may lead to destruction and alteration of the thyroid which is observed in Hashimoto's disease. This is indeed an intriguing concept of pathogenesis, although admittedly more investigation will be required before it can be finally accepted.

BLOOD

Much investigation has been directed toward an understanding of the nature and mode of transport of the circulating thyroid hormone. Using paper zone electrophoresis to separate plasma protein fractions and radioactive iodine as a tracer, it has been possible to study the behavior of iodine-containing compounds. The serum of euthyroid and hyperthyroid subjects was studied following the administration of therapeutic quantities of radioactive iodide.[5] Initially, most of the I^{131} was unbound by protein, although there was a definite progressive localization in the albumin fraction. In the normal subject (Fig. 1A), in addition to albumin-bound radioactivity, localization of radioactivity appeared after several days in an area just ahead of alpha-2-globulin where it gradually increased. The same sequence was found in the hyperthyroid subjects (Fig. 1B); however, in these the radioactivity in the inter-alpha zone appeared sooner, increased more rapidly to higher levels and declined more sharply. This more rapid turnover of I^{131} in the hyperthyroid subject suggests that the circulating hormonal iodine is carried by this alpha globulin. Further analysis of the alpha globulin I^{131} was studied in hyperthyroid subjects given therapeutic doses of the isotope, using starch zone electrophoresis.[6]

Serum taken four days after treatment was subjected to electrophoresis (Fig. 2). The

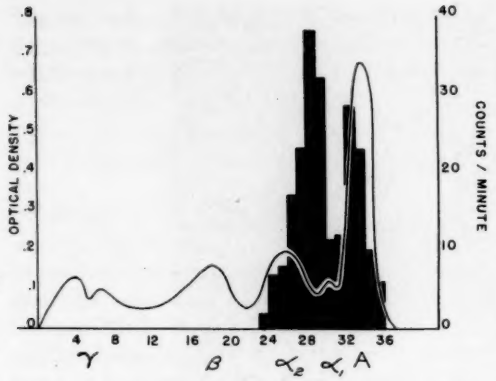

FIG. 2. Distribution of radioactivity in serum protein fractions separated by starch electrophoresis. Bar graph represents radioactivity in 1 cm. segments of starch block. Lineargraph represents protein determination in eluates of corresponding segments. From: LARSON, F. C., DEISS, W. P. and ALBRIGHT, E. C. *J. Clin. Invest.*, 33: 230, 1954.[6]

protein from the inter-alpha and albumin zones was eluted from the starch and extracted with butanol. Radioactivity in the albumin was not extractable and, under these conditions, is assumed not to contain thyroxine. That of the inter-alpha zone was extractable, and was analyzed by paper chromatography which showed that it consisted entirely of thyroxine. Triiodothyronine was not identified in the alpha zone. Since it has been shown that triiodothyronine is present in small quantities in whole plasma, the reason for its absence from the thyroxine-binding globulin when radioactivity in this zone is maximum following oral administration of the isotope is of interest. Experiments on the *in vitro* binding of labeled thyroxine and labeled triiodothyronine have shown that under these conditions both associate with the specific alpha globulin, but that thyroxine binds more firmly and can displace triiodothyronine from this binding site.[7] This explains the absence of triiodothyronine in the alpha globulin in the *in vivo* observations already cited, and may account in part for the greater speed of biological activity of triiodothyronine observed clinically. Thus, it appears that the principal circulating thyroid hormone is in fact thyroxine, and that it is transported in association with a thyroxine-binding protein found in the inter-alpha globulin area.

Subsequent experiments have provided additional information about serum thyroxine-binding protein. It has been shown to exist in plasma in quantity greater than required to bind endogenous thyroxine, for it will bind additional thyroxine when added *in vitro*. With small increments of exogenous thyroxine (0.1 μg./ml.) all is bound by the specific protein, while with larger concentrations thyroxine can be found in all protein fractions, with albumin the main secondary carrier. From evidence in an athyrotic subject it appears that the total thyroxine binding capacity of the thyroxine-binding globulin is approximately 0.4 μg./ml. About one-third of this capacity is utilized in the normal subject, while one half or more is utilized in the hyperthyroid subject.[8]

The binding is highly specific for the thyroxine molecule.[9] A number of analogues of thyroxine, including the stereoisomer d-thyroxine, have been examined with respect to their degree of binding; all were found to have less affinity than thyroxine for this protein. Diiodotyrosine, diiodothyronine and iodide did not bind at all. From knowledge of the structural formulas of the analogues tested, it appears that the amino, carboxyl and phenolic groups of the molecule are essential for the optimum binding and transport of thyroxine. Moreover, a critical spatial distribution of the binding sites on the protein is clearly indicated.

Recently, interesting alterations in thyroxine-binding capacity under specific hormonal influences have been found. Dowling et al.[10] and others have reported that a marked augmentation of thyroxine binding by serum protein occurs in pregnancy, beginning as early as the twenty-first postovulatory day, persisting throughout pregnancy and continuing for six weeks into the puerperium. Similar changes have been produced by administration of estrogens. These alterations are a part of the total thyroidal abnormality in pregnancy often consisting of thyromegaly, elevated serum protein-bound iodine, increased thyroidal I^{131} uptake and decreased rate of thyroxine utilization. Opposite effects have recently been

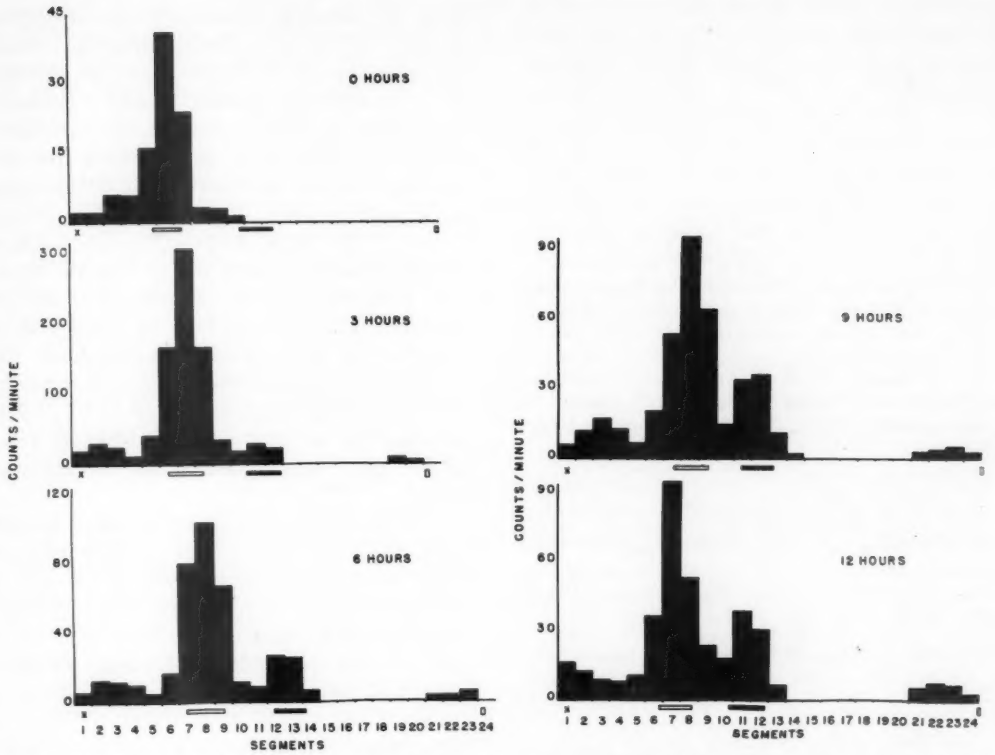

FIG. 3. Distribution of radioactivity in chromatograms following varying periods of incubation. The starting point of chromatography is indicated by X, the front by the vertical open bar. The position of thyroxine is shown by the horizontal open bar, that of triiodothyronine by the horizontal closed bar. From: ALBRIGHT, E. C., LARSON, F. C. and TUST, R. H. *Proc. Soc. Exper. Biol. & Med.*, 86: 137, 1954.[14]

observed with methyl testosterone,[11] i.e., decreased serum thyroxine-binding capacity and increased rate of thyroxine utilization. In this connection in 1944 Kinsell[12] reported an exacerbation of hyperthyroidism in patients receiving methyl testosterone therapy. The problem of the interrelationships between thyroid function and gonadal function is a promising one for future investigation.

PERIPHERAL TISSUES

One of the major problems in thyroid physiology concerns the metabolic pathway of thyroxine in the peripheral cells of the body. There has been a tremendous deficiency in our knowledge in this area although some progress is being made.

From clinical and experimental observations it has been known that the maximal metabolic effects of thyroxine occur after a latent period of several days following administration of this hormone. This delay in response may be due to transformations in the hormone which occur in the peripheral tissues. Consistent with this hypothesis is the observation that triiodothyronine, given to the animal or human subject exerts a more rapid action than thyroxine. Therefore, the removal of one atom of iodine from thyroxine to form triiodothyronine may be one step in the transformation of the circulating thyroid hormone into a form active at the cellular level. The suggestion by Gross and Pitt-Rivers[13] that triiodothyronine was derived from thyroxine by deiodination in peripheral tissues led to a useful working hypothesis; namely, that thyroxine, a slowly acting compound, has no metabolic activity except as a precursor of the more rapidly acting triiodothyronine.

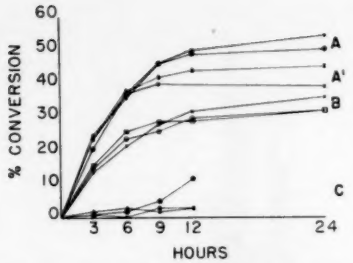

FIG. 4. Conversion of thyroxine to triiodothyronine by slices of rat kidney related to duration of incubation in (A) cold induced hyperthyroid, (A^1) desiccated thyroid-fed hyperthyroid, (B) normal, and (C) thyroidectomized animals. Each curve represents an experiment using two rats. From: LARSON, F. C., TOMITA, K. and ALBRIGHT, E. C. *Endocrinology*, 57: 338, 1955.[15]

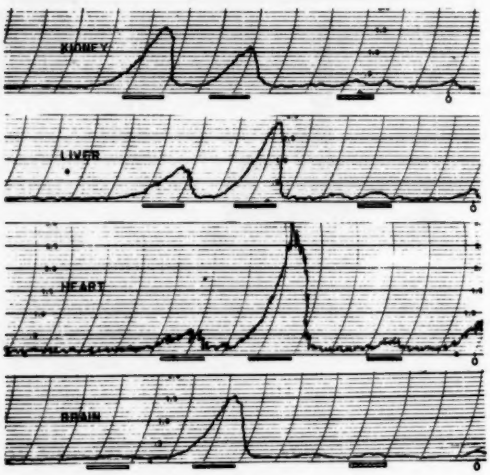

FIG. 5. Metabolism of labeled triiodothyronine by solubilized mitochondrial enzyme system of rat kidney, liver, heart and brain. Position of non-radioactive marker iodine is shown by the cross hatched bar, triiodothyronine by the solid bar, and triiodothyroacetic acid by the open bar. O = origin of chromatogram. Chromatographic front not shown. From: ALBRIGHT, E. C., TOMITA, K. and LARSON, F. C. *Endocrinology*, 64: 208, 1959.[19]

Several observations support this view. (1) Thyroxine is present in serum in greater quantities than triiodothyronine. (2) When labeled thyroxine is given to thyroidectomized mice or propylthiouracil-treated rats, labeled triiodothyronine can be recovered from the plasma and tissues. (3) Butyl-4-hydroxy-3:5-diiodobenzoate has been shown to antagonize the metabolic action of thyroxine and to enhance the action of triiodothyronine, which it does presumably by inhibiting, competitively, the deiodination process. Direct evidence in support of this concept of the metabolic pathway of thyroxine was afforded by the demonstration of the deiodination of thyroxine to triiodothyronine *in vitro* by slices of rat kidney.[14] In these studies (Fig. 3), kidneys of normal animals were used. The deiodination proceeded slowly as only 0.001 μg. of thyroxine/gm. of tissue/hour was deiodinated. The system was unusually stable under the conditions employed as some activity remained after nine hours of incubation at 37° C. The deiodinating system was heat-labile and inhibited by thiouracil, iodide and triiodothyronine.

The deiodination reaction assumes greater physiologic significance when it is demonstrated that the reaction depends on the level of thyroid activity.[15] A direct relationship seems to exist between the level of thyroid activity and the capacity of kidney slices to deiodinate thyroxine (Fig. 4). The kidney of hyperthyroid rats, in which the state of hyperthyroidism was induced either by thyroid feeding or by exposure to cold, had more active deiodinating systems than the normal animal. The kidney slices of athyrotic animals, on the other hand, were found to have almost no deiodinating activity. It is suggested, therefore, that an adaptive deiodinating enzyme exists in kidney tissue which is conditioned by the level of thyroxine in the tissue.

Studies are currently in progress in our laboratory extending some of these observations to man. Samples of kidney, liver, heart and skeletal muscle have been obtained at operation. With slices prepared from human kidney the deiodination of I^{131}-labeled thyroxine to labeled triiodothyronine has been observed repeatedly. Other tissues to date have shown no significant formation of triiodothyronine.

The administration of triiodothyronine to the intact animal or to man is still attended by a latent period of several hours. Therefore, it is logical to search beyond triiodothyronine for further transformations involved in the derivation of thyroid hormone active at the

intracellular level. In this connection the physiologic effects of triiodothyioacetic acid have been studied *in vitro*. Thibault and Pitt-Rivers[16] reported stimulation of oxygen consumption within fifteen minutes when triiodothyroacetic acid was added to slices of rat kidney. Wang and Lardy[17] have found that triiodothyroacetic acid is effective in uncoupling oxidative phosphorylation (a proposed mode of action of thyroid hormone) in heart mitochondria at about one-tenth the concentration required for triiodothyronine. It has been suggested that, because of its rapid and potent action *in vitro*, triiodothyroacetic acid may be a cellularly active form of thyroid hormone.

It was of interest, therefore, to determine whether enzyme systems capable of converting triiodothyronine to triiodothyroacetic acid are present in extra-thyroidal tissues. When I^{131}-labeled triiodothyronine was incubated with *in vitro* systems containing mitochondria of rat kidney,[18] liver and heart,[19] the principal reaction product was identified as labeled triiodothyroacetic acid (Fig. 5). Furthermore, labeled triiodothyroacetic acid has been recoverd from rat kidney and muscle after injection of labeled triiodothyronine into the intact animal. It would appear, therefore, that there are enzyme systems present in the kidney, liver and heart which are capable of carrying out this metabolic transformation of triiodothyronine.

These animal experiments have also been extended recently to include observations on the same reaction in human tissues. We have been able to demonstrate active conversion of triiodothyronine to triiodothyroacetic acid by mitochondria prepared from both human liver and kidney tissues obtained at operation.

The presence of this enzyme system in the mitochondria of tissues other than kidney, together with the earlier observation that intravascular triiodothyroacetic acid is rapidly eliminated, makes it appear unlikely that triiodothyroacetic acid is formed in the kidney for use elsewhere in the body. It appears more likely that many tissues can carry out this conversion. The presence of this enzyme system in these tissues increases the likelihood that it has a role in the metabolism of the hormone. That this role may be physiologic is suggested by the observation that tissues most susceptible to the stimulating effect of thyroid hormone (heart, kidney and liver), are most active in converting triiodothyronine to triiodothyroacetic acid. It must be emphasized that the presence of triiodothyroacetic acid in this metabolic sequence does not necessarily mean that it is the active hormone, since it may well be a precursor or product or one of many hormones yet to be discovered.

SUMMARY

In the time allotted for this discussion an attempt has been made to integrate some of the many recent developments in the investigation of thyroid physiology. This survey includes findings on the hypothalamic-hypophyseal control of thyroid gland function; the biochemical reactions in the thyroid and their alterations in certain disease states; the transport of thyroid hormone in the blood; and the metabolic fate of thyroid hormone in the target tissues of the body.

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Arteriosclerosis—The Lesion

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MOST of the modern textbooks refer to, and most of us either consciously or unconsciously think of, "arteriosclerosis" as resulting from a disturbance in the metabolism of lipids. We shall, therefore, eliminate two commonly recognized forms of arteriosclerosis, namely Mönckeberg's sclerosis and arteriolar sclerosis from this discussion and confine ourselves to atherosclerosis. We believe that the medionecrosis and calcification which occur in muscular arteries (Mönckeberg's sclerosis) have a different etiology and pathogenesis from that of atherosclerosis which occurs in the inner layers of both elastic and muscular arteries.

The ultimate point of reference and significance of atherosclerosis must be the lesions in the arterial system of man. We do not believe it is possible to have atherosclerosis without lesions. A "lesion" is defined by Dorland as "any pathological or traumatic discontinuity of tissue or loss of function of a part."

There are many ways of studying lesions; gross, microscopic, histochemical, chemical and physical. Regardless of which method is selected, most important in the study of lesions, especially the lesions of atherosclerosis, is the realization that what is being studied at any given moment is but a single film out of a long motion picture reel. Since the object of such studies is to reconstruct a true-to-life picture, the emphasis should be on the pathogenesis of the lesions, rather than the lesions themselves. A diagram depicting a concept of pathogenesis of atherosclerosis which we are now using is shown in Figure 1.

The aorta was selected as the most easily obtainable large area in the arterial system, and the unstained lesions in this vessel were studied quantitatively, qualitatively and topographically in 300 consecutive autopsies on subjects of all ages in The Charity Hospital of Louisiana, New Orleans. This was followed by a more detailed study of the early lesions in over 1,100 autopsies, aged one to forty years, from seven different geographic areas (New Orleans, England, Spain, Puerto Rico, Costa Rica, South Africa and Guatemala). In this latter study the aorta (together with the coronary, cerebral and pulmonary arteries in some cases) was stained with Sudan IV and the percentage of the intimal surface that stained red by this technic was estimated and recorded.

Gross photographs, graphs and tables* have been presented which support the following conclusions:

(1) The aorta is the first anatomic area involved by atherosclerosis, and is a reasonably good reflector of the degree of this change in the entire arterial tree.

(2) Some degree of sudanophilia (early fatty streaks) in the intima of the aorta has been found in every individual over three years of age.

(3) The degree of atherosclerosis in cases of accidental death was similar to that in cases of death due to natural causes, hence the terminal illness was not the determining factor.

(4) The most striking quantitative increase in aortic atherosclerosis occurred between the ages of eight and eighteen years, and suggested

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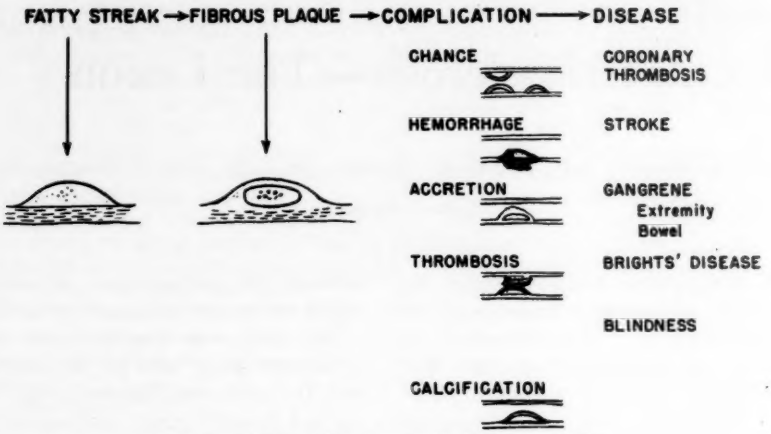

FIG. 1. Concept of pathogenesis of atherosclerosis.

a relationship of atherogenesis to the hormonal changes of puberty.

(5) Fibrous plaques have followed the fatty streaks, both quantitatively and topographically, approximately twenty years later.

(6) Evidence for reversibility was shown by fatty streaks, but not by fibrous plaques.

(7) The data viewed as a whole have not supported the current concepts of passive filtration as the major mechanism involved in atherogenesis. They have indicated active metabolic processes under the control of local and systemic factors as a more probable mechanism.

MICROSCOPIC APPEARANCE

The difficulties encountered in describing and interpreting the gross findings are multiplied many times in describing and interpreting the microscopic findings. These difficulties

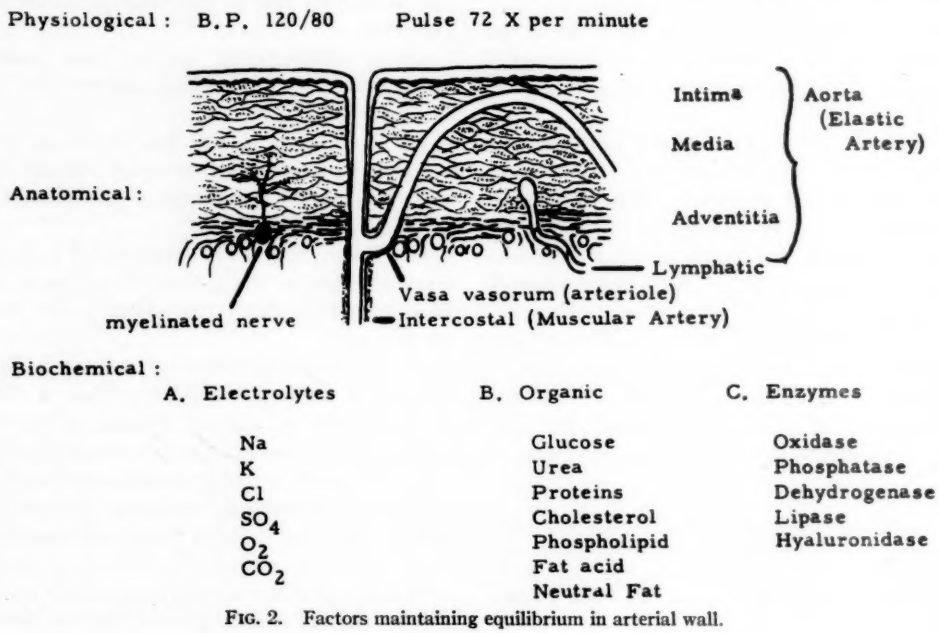

FIG. 2. Factors maintaining equilibrium in arterial wall.

increase in geometric proportion in describing and interpreting the submicroscopic findings as revealed by the electron microscope.

The obvious difficulties inherent in routine methods, fixation, embedding, sectioning and staining, are appreciated by most pathologists. The greatest difficulty arises in the selection of "representative" sections and in the reconstruction of the usual sequence of events. Serial sections are impractical, hence interpretation must be based on material selected as representative; and this is where differences of opinion arise.

Let us review some of the factors maintaining "normal" equilibrium and structure in the arterial wall. Figure 2 depicts an elastic artery (aorta) giving rise to a muscular artery (intercostal artery) which in turn gives origin to an arteriole (vasa vasorum). Some of the physiologic and biochemical considerations are also depicted. Rather than describe classic lesions in the usual descriptive terms, we have chosen to minimize special stains, simplify terminology and emphasize dynamics. The problem may be simplified by eliminating two more or less obsolete ideas about atherosclerosis: (1) Since we believe that lipid is an integral part of the lesion of atherosclerosis from the start, the Rokitansky hypothesis of "fatty degeneration" resulting from hypoxic and dystrophic changes in the depths of fibrous intimal thickenings may be disregarded for the purposes of this paper. (2) Since we have never seen a fat-laden phagocyte penetrating the endothelial lining from the lumen, the Leary hypothesis may also be disregarded.

The usual early lesion (Fig. 3) shows loose intimal thickening and lipid. The loose intimal thickening is composed of edema fluid; acid mucopolysaccharides; increased cells and increased fibers, both elastic and collagenous. The lipid is composed of neutral fats, cholesterol and cholesterol esters, and phospholipids. We are not aware of any evidence that an abnormal lipid nor any other abnormal substance is responsible for the lesion.

The usual early lesion yields evidence for an increase in formed and unformed elements including imbibition of fluid; it does not tell us whether there is any change in endothelial permeability, or whether the major portion of the increase comes from the lumen or the vasa vasorum. The answer to this latter question is simplified by always finding the early changes in the inner layers of the arterial wall, thus indicating that the increments of fluid come from the lumen.

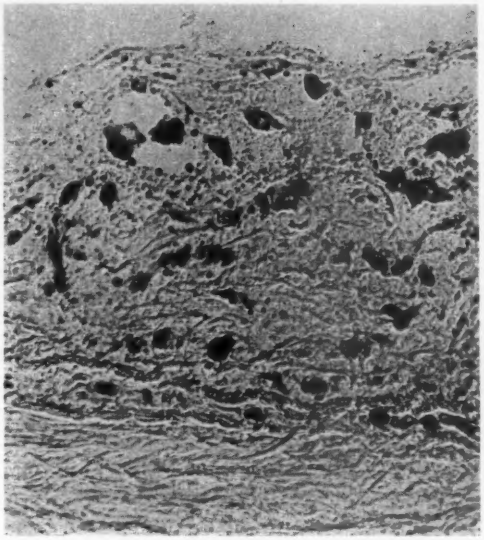

FIG. 3. Usual early atheromatous lesion (fatty streak) showing intracellular and lumpy extracellular lipid in an area of loose fibrous intimal thickening. Sudan IV stain, original magnification, X 350.

The components of the usual early lesion do not answer the main question—which comes first the lipid or the changes in the ground substances? Does the lipid alter the ground substance or does the altered ground substance trap lipid?

Let us examine the various possibilities: increased filtration from the lumen; increased local formation; and decreased local removal. We are witnessing local accumulation which could result from any discrepancy in the rates of the various processes that favor local accumulation.

Thus far in every patient beyond the age of three, the aorta has shown some degree of focal sudanophilia. Therefore, we cannot keep from asking certain questions about the definition of "normal." Defining the rates of turn-over of lipids, lipoproteins and muco-

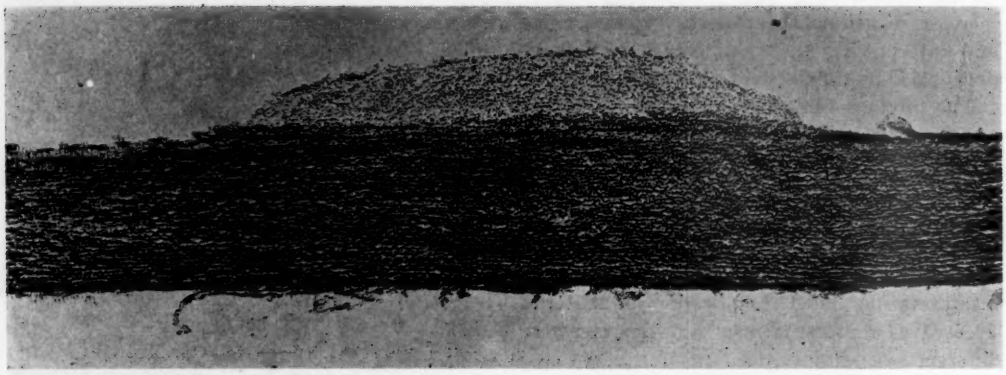

FIG. 4. Loose edematous intimal "mound" without lipid. Sudan IV stain, original magnification, X 50.

polysaccharides, constantly taking place in the arterial wall, may prove more valuable than defining and describing the accumulation.

We have described, illustrated and enumerated the formed and unformed elements that compose the usual early lesion of atherosclerosis. Now we will describe some of the unusual findings and attempt to relate these to the usual findings in order to obtain a better grasp of the entire process.

A "hillock" or "mound" of loose intimal thickening without lipid is rare. Such a lesion occurred in a ten year old boy who died of ulcerative colitis and amyloidosis (Fig. 4). Serial sections of this lesion have not been made, hence the possibility of a minimal amount of stainable lipid somewhere along the course of the lesion cannot be excluded. This particular lesion was translucent and gelatinous instead of opaque and yellow (or red after the application of the Sudan stain). Hence perspective was maintained and there is reason to believe that this section of this lesion is representative. The absence of stainable lipid, however, does not tell us whether: (1) lipid was present at some previous time and was carried away; (2) lipid is present in masked form; or (3) such lesions without lipid really occur. However, we are reasonably certain that such a lesion will not be or become responsible for subsequent disease. It is theoretically possible that myriads of such wheals could diminish the blood supply to vital areas and precipitate disease but the term atherosclerosis would not be strictly applicable to this process. We would rather defend the original concept of pathogenesis of atherosclerosis: No lipid—no fatty streak; no fatty streak—no fibrous plaque; no fibrous plaque—no complication; no complication—no disease.

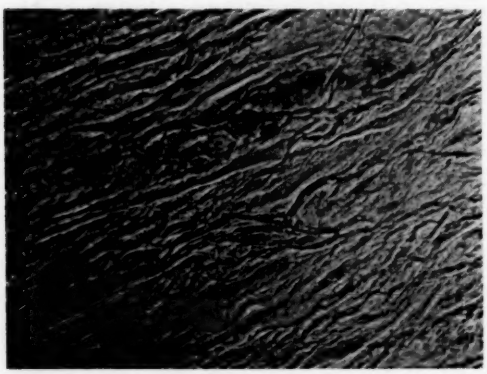

FIG. 5. Sudanophilic granules (faint dark granular area in upper right portion of photomicrograph) in smooth muscle cells in middle third of media. Sudan IV stain of frozen section. X 350.

In rare instances routine histologic and histochemical examinations will disclose isolated foci of sudanophilic material in the aorta, which appeared normal on gross examination. This sudanophilic material appears to be intracellular, and careful histologic study shows some of these lipid-containing cells to be smooth muscle cells. This is illustrated in Figure 5 which shows fine sudanophilic granules in cells in the middle third of the media of the aorta. The illustration is

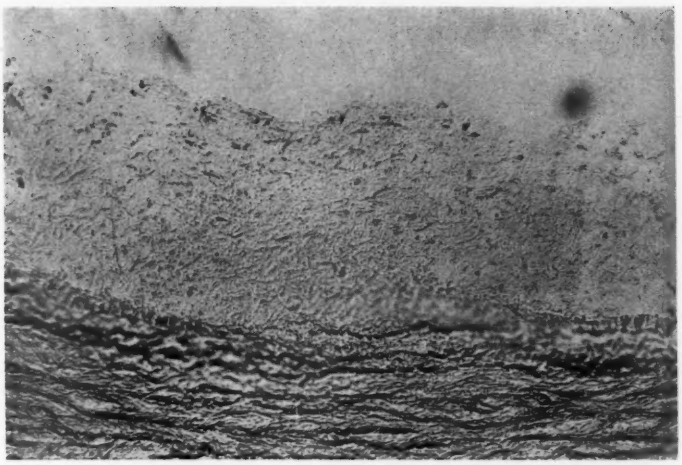

FIG. 6. Scattered intracellular lipid (black) in "mound" of loose edematous intimal thickening. Little or no extracellular lipid. Sudan IV stain of frozen section. X 350.

poor because even with optimum use of the condenser and fine adjustment of the microscope it is difficult to identify the cells positively. The evidence that these cells are smooth muscle cells is convincing when viewed in perspective, and we shall adduce additional evidence of this latter.

What significance could be attached to the chance findings of fine droplet sudanophilic material in the cytoplasm of smooth muscle cells? It is difficult to imagine this as being due to filtration. It could be fatty degeneration, but what does this term mean when it is applied to a "normal" aorta being studied incidentally for the natural history and geographic pathology of atherosclerosis? To us it suggests the possibility of a metabolic activity and some morphologic confirmation of this has been found in the few electron micrographs that have been prepared in our laboratory, namely the finding of osimophilic cytoplasmic globoid bodies that do not correspond to any of the known organelles or other cytoplasmic structures (mitachondria, Golgi apparatus and cytoplasmic reticulum) that have been described for normal smooth muscle cells.

Another unusual finding in the routine study of early aortic lesions is the presence of oose intimal thickenings with predominantly intracellular lipid (Fig. 6). We do not know what these are but some of the cells are stellate and resemble fibroblasts, some of them are spindle-shaped and resemble smooth muscle cells, and some of them are round or ovoid and resemble phagocytes. Of particular interest is the fact that most of them appear to be fixed tissue cells and not wandering cells.

Still another unusual finding is a more diffuse area of intimal thickening in which spindle shaped cells run parallel to the surface and appear indistinguishable from smooth muscle cells in the media (Fig. 7). The majority of these cells contain finely divided sudanophilic material and only in a few scattered foci does the sudanophilic material appear to be extracellular (Fig. 8).

How are we to interpret these unusual findings? It is easier to assume that the cytoplasmic lipid in these cells originated *in situ*, presumably under the influence of some metabolic stimulus, than it is to imagine that lipid containing phagocytes has undergone metaplasia to fibroblasts and/or to smooth muscle cells without first having lost the sudanophilic material contained in its cytoplasm.

The exact designation of these cells is not a primary concern. It suffices to refer to them as mesenchymal cells of the arterial wall with multiple potentialities, i.e., of becoming fi-

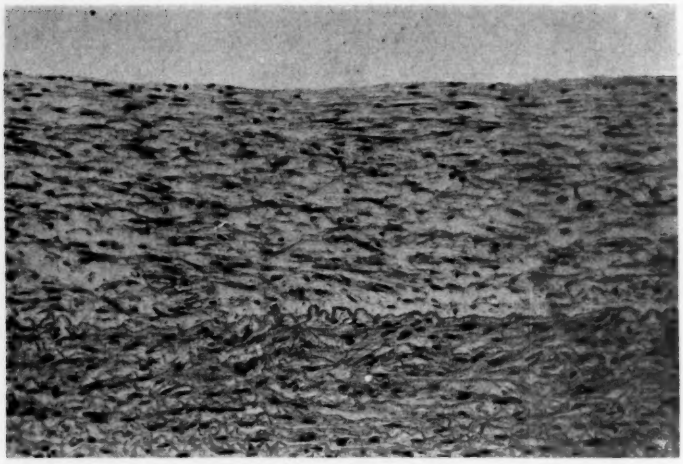

FIG. 7. Loose intimal thickening (the internal elastic membrane is seen faintly near the junction of the middle and lower thirds of the photomicrograph) with numerous spindle shaped cells (smooth muscle?) running parallel to the surface. Hematoxylin and eosin stain. X 250.

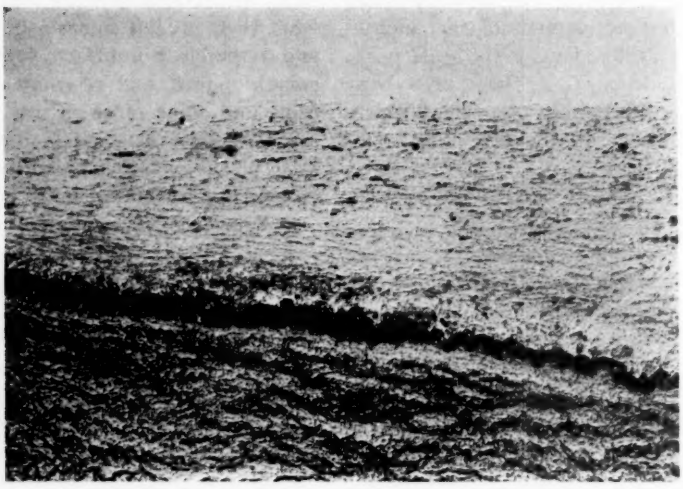

FIG. 8. This is a Sudan IV stain of a frozen section of the lesion seen in Figure 7 and is approximately three times as thick, which explains the increased cellularity and density. This thick frozen section shows that many of the spindle shaped cells in the intima contain sudanophilic (black) granules. X 250.

broblasts, smooth muscle cells or phagocytes. We can imagine some of these fixed tissue cells (the mesenchymal cells of the arterial wall) undergoing metabolic upsets with the development of sudanophilic granules in their cytoplasm, then undergoing death or attrition of some sort with liberation of their lipid into extracellular spaces, and other mesenchymal cells proliferating to form phagocytes to engulf and attempt to remove the lipid.

Since we have never seen fat-laden phagocytes passing through or between endothelial cells, and since we have seen intracellular lipid

without extracellular lipid but never the reverse, we believe that the fixed tissue cells in the arterial wall are capable of forming sudanophilic granules that may be extruded into extracellular spaces, to be taken up again by phagocytes (derived from proliferation of other mesenchymal cells), and so on in sequence until the lesion is reversed or until it becomes walled off in a lipogranulomatous process that converts the early fatty streak into a fibrous plaque with vascularization at its base and margins. What has surprised us from a study of these early lesions is the significant number of mesenchymal cells in the inner layers of the blood vessel wall which appear to be smooth muscle cells. It is our belief that progress in unravelling the mysteries of atherogenesis in man will vary with our knowledge of the local and systemic factors which govern the metabolism of these mesenchymal cells.

Whether or not the rare loose edematous intimal lesions without lipid are due to the same stimulus is not known. It is not difficult to imagine that these lesions represent a response to injury (metabolic or otherwise) and that at the time of the injury the precursors for lipid either were not available or were being diverted into other pathways. The healing of such lesions, as well as the lesions from which lipid has been removed, would explain the eccentric fibrous intimal thickenings that are so commonly observed in various parts of the vascular system. These thickenings have tempted Rokitansky and others to postulate hypoxic fatty degeneration in the depth of these fibrous intimal thickenings as the method of atherogenesis. In our opinion these lesions are more closely allied to scars that accumulate with the passage of time and, to the best of our knowledge, they are never related to clinical disease. No lipid—no disease.

Before we review the later stages of the lesions of atherosclerosis, there is one feature of the early lesions that we would like to emphasize. We believe that this feature, which we shall refer to as "dispersion of lipid," offers one of the greatest hopes of ultimate control of the process and prevention of atherosclerosis. In Figure 9 we see not only classic intra- and extracellular lipid but also finely divided dust-like particles of sudanophilic material in the background. This is extracellular lipid, but we have never seen it in the absence of the other two classic forms in which lipid occurs, namely intracellular and "lumpy" extracellular. If this dispersed lipid—whose particulate size is in the range of chylomicrons and whose appearance simulates the results obtained in some of the *in vitro* perfusions of various types—were common and could be found sometimes in a diffuse form proceeding from endothelium to internal elastic membrane without admixtures of the other two forms of lipid, we would be much more tolerant of the concepts of atherogenesis that emphasize "too much in the diet," "too much in the blood," "too much in the blood vessel wall" and imply filtration as the basic mechanism of atherogenesis. However, since we have never

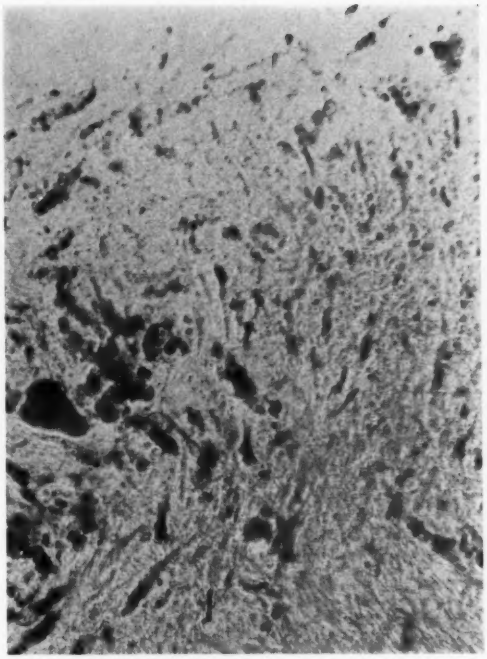

FIG. 9. Loose intimal thickening (the endothelium is seen in the left upper corner) with intracellular, lumpy extracellular, and "dispersed" lipid. The latter is seen faintly in the background in the right half of the photomicrograph. Sudan IV stain of frozen section. X 600.

FIG. 10. Loose fibrous intimal thickening with intracellular and extracellular lipid and "dispersion of lipid" about finely frayed elastic tissue. Sudan IV stain of frozen section. X 250.

seen this dispersed lipid alone, as already stated, we must deduce that it is a secondary phenomenon and not a primary phenomenon. The fact that it does occur frequently offers a *modus operandi* for reversibility and a hope that some way may be found to maintain or to encourage clearance of waste products and thus prevent the accumulation which undergoes spontaneous combustion in the form of coronary occlusion, apoplexy and gangrene in a certain percentage of cases at some later time.

We are looking for an explanation as to the nature of the process whereby large or "lumpy" masses of extracellular fat may become dispersed, or the accumulation of liberated intracellular fat may be prevented. We know that in other parts of the body emulsification of lipid is prerequisite to its passage across cellular and tissue barriers. The nature of this process is intriguing. It is possible that detailed study of the cases in which emulsification occurs will enable us to narrow the field in which to look for something specific.

In the discussion of the lesions thus far little has been said about elastic tissue and the internal elastic membrane. We believe that the physical barrier imposed by this tissue has been overemphasized in the past. In fact, we have been impressed by (1) the difficulty of defining the internal elastic membrane in many of the aortas in patients under ten years of age; (2) the frequent appearance of delicate elastic fibrils in the intimal thickenings; (3) the intimate association between elastic tissue and smooth muscle cells as seen by the electron microscope; (4) the ease with which one or more condensations of elastic fibrils appear beneath and about the margins of fatty streaks; and (5) the many other vagaries of elastic tissue that do not seem to be fundamental to atherogenesis (Figs. 10 and 11).

We do not mean to leave the impression that elastic tissue plays no role in atherosclerosis, evidence of an affinity between elastic tissue and lipid has been seen, but most of the changes in elastic tissue that we have encountered belong to the reparative process rather than to atherogenesis.

Now for a brief review of the succeeding stages in the evolution of the lesions depicted in Figure 1. The distinction between fatty streak and subsequent stages is important. Recognition of this will do much to dispel confusion about atherosclerosis. None of the changes described thus far is associated with clinical disease; yet without the changes that have been described clinical disease does not

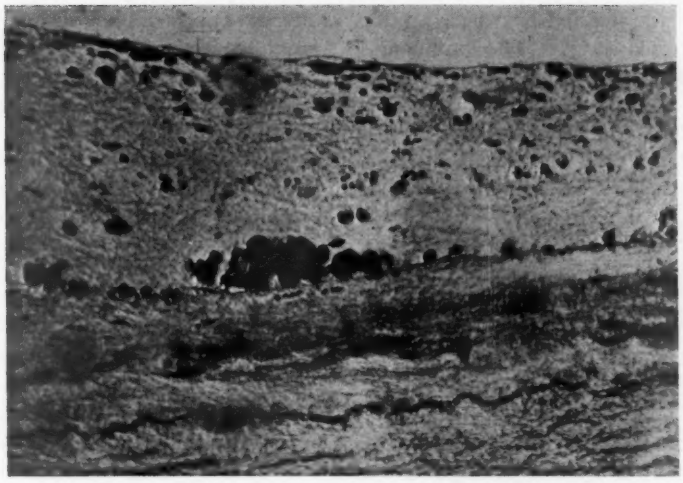

FIG. 11. "Musculo-elastic" layer containing dispersed lipid (see text). Sudan IV stain of frozen section. X 250.

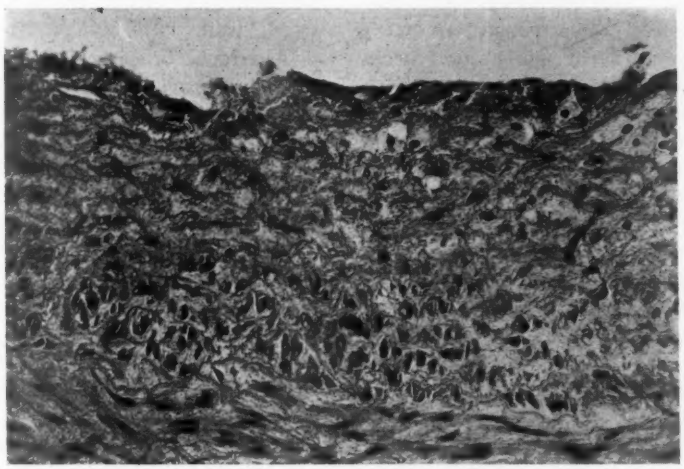

FIG. 12. Incrustation of acidophilic material (top center) over an area of intimal thickening containing foam cells. Hematoxylin and eosin stain. X 250.

ensue. All pathologists are familiar with the marked discrepancy between lesions and clinical disease. These range all the way from extreme anatomic curiosities without disease (e.g., the lengthened, tortuous, inelastic aorta with calcific distortions and gnarlings) to comparatively minimal lesions responsible for death (e.g., thrombotic occlusion over a relatively insignificant lesion in a main coronary artery). This discrepancy between lesions and clinical disease merely reflects the great factors of safety that the cardiovascular system possesses and in no way invalidates the assumption that if there had been no lesions there would be no disease.

A sharp line of distinction must be drawn between atherogenesis and the subsequent evolution of lesions that may or may not precipitate disease, for the factors involved in the evolution of lesions beyond the stage of fatty streak may be entirely different from the factors that initiated the fatty streak.

What then is the method by which a fatty streak is converted into a fibrous plaque? The incrustation of acidophilic material (that takes some of the stains for fibrin) on the endothelial surface, the incorporation of this acidophilic material into the intima by overgrowth of endothelium, and the organization (by slow fibrous tissue replacement) of this material into fibrous tissue is the common method for conversion of fatty streak to fibrous plaque (Fig. 12). This gives the inner surface of the lesion a pale gray appearance through which can be seen the sudanophilic material in the base. It is easy to see how this process can be repeated and how the resulting lesion, when detected in the later stages, could give the impression that the intimal thickening led to hypoxia in the deeper layers with ensuing fatty degeneration due to lack of metabolites including oxygen. This represents faulty reasoning due to lack of proper perspective. It is possible that such fibrin incrustation could occur on a normal area of the intimal lining of the arterial wall, but we have never seen this happen. In our experience it has always occurred over or about the margins of a fatty streak. The dictum, no fatty streak—no fibrous plaque, will hold in the majority of cases.

In addition to the conversion of fibrin deposited on the surface to fibrous tissue, the altered lipids in the intima excite a lipogranulomatous reaction which yields a fibrous capsule about the central core of necrotic debris. The capsule is rich in lipids, especially cholesterol and cholesterol esters. This results in the usual conversion of fatty streaks to fibrous plaques beyond which a variety of complications can ensue, including the superimposition of that which started the process.

SUMMARY

A concept of pathogenesis of atherosclerosis is presented in which atherogenesis has been sharply delineated from the subsequent evolution of lesions to the stage of clinical disease.

Basic to atherogenesis are active metabolic processes, presumably under the control of both local and systemic factors, on the part of mesenchymal cells in the inner layers of the arterial wall.

Occurrence of early lesions in all subjects beyond the age of three years and the persistence of these lesions in a potentially reversible form for a period of many years has indicated that the problem is basically a pediatric one.

A *modus operandi* for reversibility, namely "dispersion of lipid," is illustrated; and a hope for fundamental control of the process is sounded.

The Lipids in Relation to Atherosclerosis

RALPH T. HOLMAN, PH.D.*

THE USUAL definition of atherosclerosis suggests that the disease is a fatty degeneration of the arterial wall. It is becoming increasingly apparent that the disease accompanies abnormalities in the metabolism of various lipids, and the number of lipids involved in the process seems to be growing as investigators delve into the matter. At this stage of our knowledge, it cannot be stated dogmatically that abnormalities of lipid metabolism are the *cause* of the disease. Such may be the case, however, for imbalances induced in lipid metabolism have led to lesions resembling atherosclerosis in many respects.

The number of lipid substances in nature is legion, and most of these are not well characterized. The variety of structures is great, and their single common property is that they occur together because of similar characteristics of solubility. The state of affairs in lipid chemistry is rather parallel to that of protein chemistry. We know the structures of many of the simple units which together make up the complex lipids, and we are continually finding new units previously unsuspected within the lipid family. The compositions of the more complex lipids are known only empirically, and the detailed structures have yet to be worked out for many compounds with molecular weights of less than 1,000. The structures of proteolipids and lipoproteins are not known, and for the present we must be content with knowing the approximate composition of these substances.

Even the determination of composition of many of the mixtures or concentrates that biochemists isolate presents a difficult problem. Thus far, there is no good method for determining the triglyceride content of a lipid, and until recently the common fatty acid, oleic acid, had never been measured directly—only by difference. We have only a few chemical or physical methods which allow us to determine specific lipid components. We are accustomed to group analyses such as saponification number, iodine value, Lieberman-Burchard reaction, spectrophotometric analyses for polyunsaturated fatty acids and lipid-soluble phosphorus.

Although this condition persists in our field of of work, some progress is being made. Lipid substances differ not so much by possession of different specific structures as by being homologs, isomers or vinylogs of each other. To distinguish such minor differences, physical methods have proved to be more effective than chemical methods. Through partition, displacement and paper chromatography we are now able to separate fatty acids differing by as little as one carbon atom, a double bond or by isomerism of several types. Countercurrent distribution and fractional distillation are used for determination of composition and isolation of significant quantities of highly pure substances, but none of these more precise methods has become common practice.

Progress in biochemistry can be made only in proportion to the precision of its methodology. Thus, the rather primitive state of lipid analyses precludes a highly detailed knowledge of the role lipids play in atherosclerosis. Nevertheless, information gained by epidemiologic, biologic, nutritional and crude chemical studies indicts the lipids as being involved in atherogenesis. If we wish to approach the ultimate answer by another magnitude of detail, we must adopt stricter criteria of purity and we must develop and use modern methods

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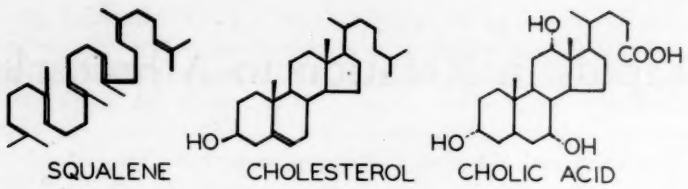


FIG. 1

which have another magnitude of precision. To state this another way, "we must not pick fleas with boxing gloves."

The following will survey the lipid substances briefly and point out known or suspected connections which specific lipids may have to the problem of atherosclerosis. This will necessitate elimination of entire areas of the lipid field, and emphasis will rest on specific substances which are implicated more than others.

THE STEROIDS

This class of lipids has been implicated in atherogenesis largely because of the presence of cholesterol in the fatty plaques. Its importance in the problem of atherosclerosis may be exaggerated by the happenstance that it is the only serum lipid which can be easily determined by a color reaction. Cholesterol, its precursor squalene, and its chief metabolic product, cholic acid, are shown in Figure 1.

For a long time it has been known that a relationship exists between the level of plasma cholesterol and the incidence of atherosclerosis and coronary heart disease. It is not known whether hypercholesterolemia is the cause of the atherosclerotic lesion or whether it merely accompanies the lesion, but, the plasma cholesterol content is a useful index of susceptibility to circulatory accidents. Moreover, feeding cholesterol to either rabbits or chickens induces lesions of their aortas which resemble atherosclerosis. This experimentally-induced atherosclerosis is the starting point for many of the tests of therapeutic value of substances.

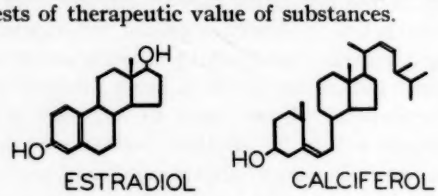


FIG. 2

Cholic acid, a metabolic product of cholesterol, is excreted in the bile. If the cholic acid content of the plasma is raised, it is accompanied by an increase in plasma cholesterol. Feeding cholic acid thus increases cholesterol transport. If cholic acid is fed with an otherwise fat-free diet, essential fatty acid deficiency rapidly develops (*vide infra*). Hypercholesterolemia, induced by cholic acid, may lead to atherosclerosis as experiments of Fillios et al.[1] suggest.

Plant sterols fed to animals and man have been reported to lower plasma cholesterol. The therapeutic effect is not great, the plasma cholesterol levels being diminished 5 to 25 per cent by the treatment. This effect has been presumed to be the result of decreased absorption of cholesterol although this explanation has been questioned.[2]

The estrogens have been found to prevent experimental atherosclerosis. Estradiol (Fig. 2) and a variety of other substances possessing estrogenic activity, have been found by Katz and other workers[3] to reduce the atherosclerotic lesions in the aorta of chickens fed an atherogenic diet containing cholesterol. Thus, hormone balance may help explain the sex difference in susceptibility of man to atherosclerosis. However, the mechanism of this action is not known.

Vitamins D, one of which is shown in the formula in Figure 2, are derived from or related to the steroids. The steroid nucleus, ruptured in ring 2, carbon 19, becomes a methylene group. Vitamins D are not obviously involved in lipid metabolism, but their function in regulating calcium metabolism suggests that these lipids may play a role in the calcification which often accompanies the development of atherosclerosis.

Vitamins A, E and K have a superficial structural resemblance in that they all have an aro-

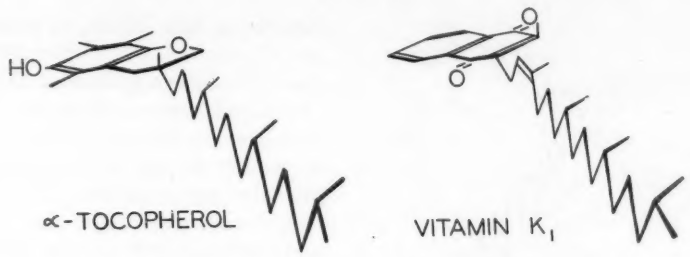


FIG. 3

matic ring system with a long branched hydrocarbon chain. Vitamins E and K, shown in Figure 3, are capable of oxidation-reduction at the quinoid nucleus. In vitamins E and K, polar groups are found in the ring system while in vitamin A the polar group is at the end of the hydrocarbon tail. All these substances are thus surface-active agents and probably find their function at a lipid-water interface.

Vitamin K is known to promote the formation of prothrombin, and inasmuch as it is involved in the coagulation of blood, it plays a minor role in atherogenesis.

The involvement of vitamins A and E is the prevention of atherogenesis is suggested by the work of many laboratories. Vitamin E (tocopherol) is known to be antioxidant *in vitro*, preventing the development of rancidity of the unsaturated fats. There is also reason to believe that tocopherol acts as a biological antioxidant. The experiments performed in Dam's laboratory[4] have shown that when animals are fed a tocopherol-free diet containing easily oxidized fat, the depot fats contain peroxidized unsaturated fatty acids. Glavind et al.[5] found lipoperoxides in atherosclerotic aorta but not in normal aorta. This suggests that the reserve of biological antioxidants of the atherosclerotic subject is low. Weitzel et al.[6] found that tocopherol had a weak antiatherosclerotic effect on hens at a dose level of 50 mg./day. The administration of vitamin A, 7 mg./day, notably improved the atherosclerotic lesions. The effect was even greater when the two vitamins were given together. Recently Hill and Larson,[7] at our institute, found that in six of eight young swine which were fed a tocopherol-free diet for fifty days or more sclerotic lesions developed in the aorta. Histologic examinations of the plaques by Dr. Jesse Edwards of the Mayo Foundation revealed the lesion to be medial calcification. All this evidence points to the involvement of tocopherol, and perhaps vitamin A, in the complicated pattern of abnormality which results in atherosclerosis.

THE PHOSPHOLIPIDS

Phospholipids also exist in great variety. They differ in the fatty acids and bases found in their structures. In Figure 4, a lecithin molecule is pictured. The lecithins contain choline esterified with a phosphatidic acid which has an unsaturated fatty acid esterified at the α' position of the glycerol and a saturated fatty acid at the β position. The phosphatidyl ethanolamines (cephalins) are analogous to the lecithins except that their base is ethanolamine. In the phosphatidyl serines, the amino acid serine replaces the nitrogen base. Phosphoinositides are known in which inositol replaces the nitrogen base. Sphingomyelins are complexes in which the base sphingosine is esterified with one fatty acid and phosphoryl choline. In the acetal phosphatides the fatty acids of phosphatidyl ethanolamine are replaced by one fatty acid, aldehyde. Most of these substances have not figured in the discussions of atherosclerosis, probably because they are not well

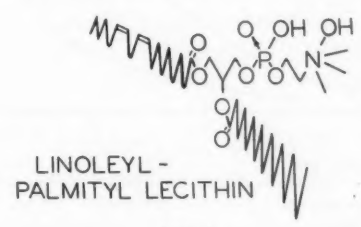


FIG. 4

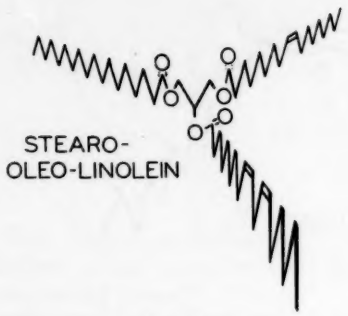


FIG. 5

known, the methods for their determination being primitive. Thromboplastin, the coagulation factor of tissue and platelets, contains cephalin as a necessary component. Thus the phosphatidyl ethanolamines may be involved in the mechanism of atherogenesis.

The phospholipids are known to be universal components of tissue structure and circulating lipoprotein. Alterations of the content of the essential fatty acid components have been shown to be induced by changing the dietary fat. Such changes in fatty acid composition, or changes in the amount of phospholipid, can alter the physicochemical properties of the circulating lipid leading to abnormal transport or utilization of fat. Generally speaking, when lipemia occurs in an animal, the phospholipids, cholesterol esters and neutral fat increase in proportion. Therefore, if the reserve of one component of any of these lipids is low in the animal it may be depleted by a lipemia induced by ingestion or synthesis of another component, and the composition of the lipoprotein may become abnormal.

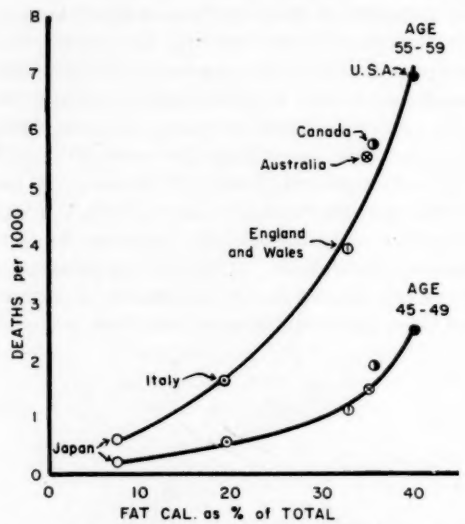


FIG. 6. Relationship between death rate from coronary heart disease and fat content of the diet. From: KEYS, A. Atherosclerosis: a problem in newer public health. *J. Mt. Sinai Hosp.*, 20: 118, 1953.[8]

THE GLYCERIDES

The most important of the lipid groups from the point of view of abundance are the glycerides, which comprise more than 95 per cent of the common vegetable oils and animal depot fats. These substances can exist in great variety because of isomerism and the larger number of fatty acids which are present in these molecules. The natural fats and oils are mixtures of mixed triglycerides. Triglycerides cannot be determined easily, either as a group or as individual substances. Thus far, the glyceride composition of no natural fat or oil has been determined farther than to state the approximate content of the groups of saturated and unsaturated glycerides. Only a few pure glycerides of known structure have been isolated from natural sources. Obviously our knowledge of this group of rather simple substances is extremely primitive, and this is a field which needs new methodology.

The triglycerides are non-polar substances, insoluble in water. The triglyceride molecule shown in Figure 5 represents one containing three of the more common fatty acids, stearic, oleic and linoleic acids. If one or two of these fatty acids are removed by hydrolysis, the resulting di- and monoglycerides have properties much different from the triglyceride. The inherent polar groups of the triglyceride are masked by the long hydrocarbon chains, but in the di-and monoglycerides the polar hydroxyl groups are exposed. This gives these substances great surface activity, and they become excellent emulsifying agents which facilitate the digestion and absorption of fat. Classwise, the triglycerides, and the di- and monoglycerides derived from them, have important nutritional functions but no specific

function is known for any specific individual glyceride.

From epidemiologic investigations, Keys and co-workers[8] have concluded that the fat content of the diet, apart from its cholesterol, bears a direct relationship to the incidence of coronary heart disease (Fig. 6) and the plasma cholesterol level of the population. These data imply that excessive ingestion of triglycerides predisposes one to the development of atherosclerosis. This hypothesis has guided the thinking of many workers, but in my opinion a closer look at the problem will reveal that the composition of the triglycerides is an important factor.

FATTY ACIDS

Fatty acids occur as components of almost all lipid structures, and the wide range of properties of the fatty acids contribute to the wide range of properties of lipids. The fatty acids themselves differ in respect to chain length, number and position of double bonds, branching of the chain and other minor variations. The state of development of analysis for individual fatty acids is far in advance of that for other classes of lipids. Displacement chromatography,[9] paper chromatography,[10] gas phase chromatography[11] and countercurrent distribution[12] can be used for the sharp separation and measurement of closely related fatty acids. An entire new range of biochemical problems can now be attacked by the newer methods.

Formulas for a group of fatty acids is shown in Figure 7.

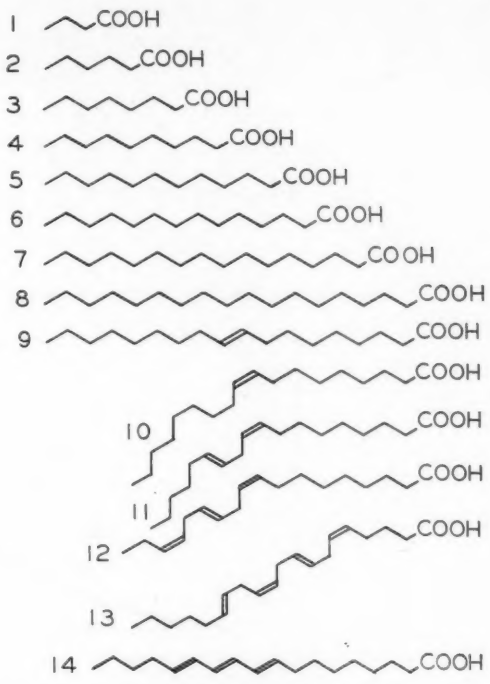


FIG. 7. Formulas for some of the common fatty acids. (1) butyric acid, (2) caproic acid, (3) caprylic acid, (4) capric acid, (5) lauric acid, (6) myristic acid, (7) palmitic acid, (8) stearic acid, (9) elaidic acid (*trans* isomer of oleic acid), (10) oleic acid, (11) one isomer of linoleic acid, (12) one isomer of linolenic acid, (13) one isomer of arachidonic acid, (14) one isomer of eleostearic acid, a conjugated unsaturated acid.

Dietary fatty acids bear some relationship to the blood-clotting process. Lipemic blood is found to be more coagulable than non-lipemic blood and is conducive to fat embolism.[13] Lipemia caused by saturated fat reduces the activity of fibrinolysin[14] but lipemia caused by unsaturated fats does not. Poole[15] found that free fatty acids added to plasma reduced the clotting times, and his experiments suggest some differences in effect dependent upon the fatty acid added. Davis[16] has found that gellation time of serum albumin is influenced by the type of free fatty acid added. Saturated acids reduce the gellation time more than do the unsaturated acids. Thus, prolonged or continual lipemia is a hazard which increases susceptibility to circulatory accidents. This phenomenon should be studied more closely, attention being paid to the effects of different pure fatty acids.

Bronte-Stewart[17] measured the extent and duration of lipemia following a fatty test meal given to persons who had had myocardial infarction. For one week thereafter the patients were given corn oil in addition to their regular diet. The fat tolerance curve was measured again and the lipemia was not as severe nor did it remain as long as in the previous test. It is therefore clear that the lipemia following a fatty meal can be moderated by the type of fatty acid ingested.

In our laboratory it has been found that the requirement of essential fatty acids, is increased

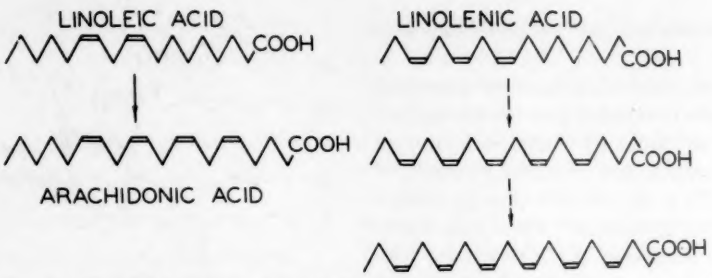


FIG. 8. Simplified pattern of metabolism of polyunsaturated acids.

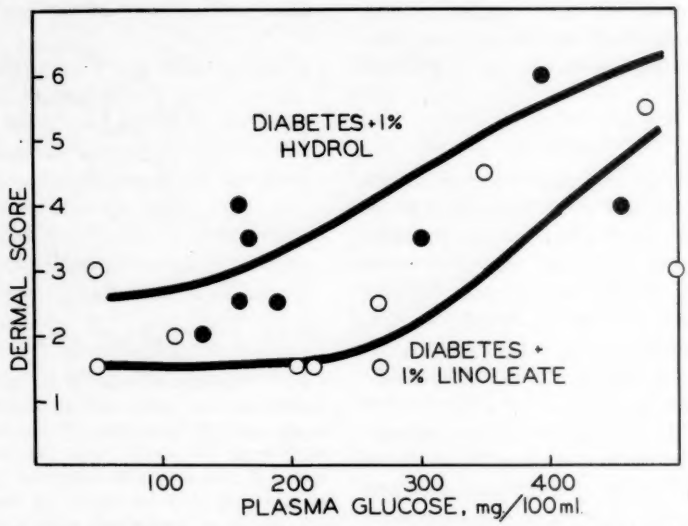


FIG. 9. The effect of diabetes upon dermal symptoms of essential fatty acid deficiency. From J. J. Peifer and R. T. Holman, unpublished data.

as the content of saturated fat is increased in the diet.[18] This suggests that the proportion of essential fatty acids to other fatty acids in the diet may be as important as the absolute amount of essential fatty acids present. The high fat intake of Americans is predominantly of the saturated type of fat, whereas the fat content of the low fat diet of the Japanese or the Bantu is proportionately more unsaturated. Hence the problem of total fat intake may be a problem of the type of fat eaten.

Most of the fatty acids have no known specific function. However, some of the unsaturated acids with two or more *cis* methylene-interrupted double bonds have been shown to be required by animals as dietary nutrients. The discovery of the essentiality of these fatty acids was made by Burr and Burr[19] in 1929, and in succeeding years these substances have been the subject of much investigation.[20] The "essential fatty acids" are shown in formula in Figure 8. Linoleic and arachidonic acids, which have identical hydrocarbon ends on their molecules, provide both restoration of growth and recovery from skin symptoms in animals made deficient of essential fatty acids by feeding them a fat-free diet. Linolenic acid and presumably those acids which have identical hydrocarbon ends on their molecules provide only the growth function. Many polyunsaturated acids existing in nature have not yet been isolated in pure form or tested for essential fatty acid activity.

The methylene group flanked by two double

bonds is reactive and it is this structure which provides special chemical properties and the physiologic activity of these substances. The non-conjugated double bonds can be isomerized by alkali at high temperatures to yield conjugated forms which can be identified specifically by their ultraviolet spectrums. This is the basis of the best current method for the analysis of the polyunsaturated fatty acids.[21]

The active methylene group is also easily attacked by oxygen and it is this oxidation that is the cause of rancidity in fats and oils. Autoxidation can presumably take place *in vivo*, for lipoperoxides have been found in tissues of vitamin E-deficient animals.[4] Glavind et al.[5] found that lipoperoxides exist in the atherosclerotic aorta but not in the normal aorta, and that the severity of the lesions parallels the peroxide value of the aorta lipid.

The polyunsaturated acids occur in a large proportion of the cholesteryl esters of plasma. Thus, they are circumstantially involved in cholesterol transport and metabolism. Cholesteryl esters accumulate in the livers of rats deficient in essential fatty acids and the esters become more saturated. The relationship of essential fatty acids to cholesterol transport also has been inadvertently pointed out by some experiments from our laboratory.

In an effort to accelerate essential fatty acid deficiency, means were sought to increase lipid turnover. Our first experiments[22] tested the effect of diabetes upon the onset of essential fatty acid deficiency. Alloxan diabetes in rats fed an essential fatty acid-free diet induced severe symptoms of deficiency within one month rather than the usual three months required on a fat-free diet alone. Figure 9 shows that the more severe the diabetes, the more severe the symptoms of essential fatty acid deficiency at the end of one month. Rats which received linoleate were only partially protected from the essential fatty acid deficiency.

Because of the known association of essential fatty acids and cholesterol in the circulating cholesteryl esters, it was suspected that the accelerated deficiency was caused by the hypercholesterolemia which accompanies diabetes. Therefore, rats were fed a fat-free diet containing 1 per cent cholesterol, and a similar acceleration of essential fatty acids deficiency was observed. This is shown in Figure 10 in which the onset and cure of cholesterol-accelerated deficiencies are traced.

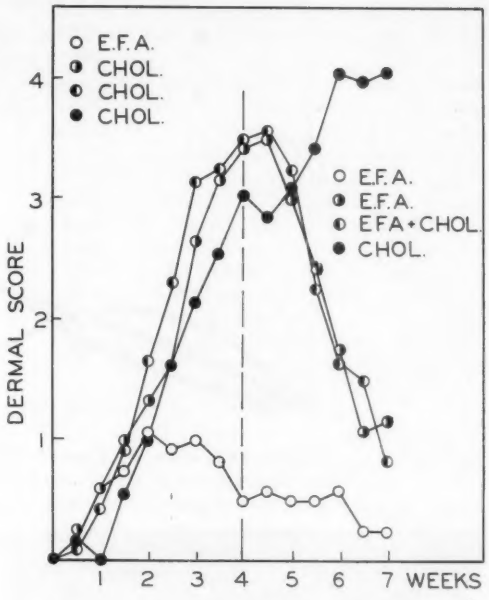


FIG. 10. The effect of dietary cholesterol (1 per cent) upon dermal symptoms of essential fatty acid deficiency in young rats. From: HOLMAN, R. T. and PEIFER, J. J. Acceleration of essential fatty acid deficiency by dietary cholesterol. *J. Nutrition* (in press).[25]

Hypothyroidism, which also is accompanied by hypercholesterolemia, was induced in rats fed a fat-free diet.[18] Again the deficiency appeared within a month. The accelerated deficiency was so severe that it could not be prevented fully by doses of linoleate usually adequate to prevent essential fatty acid deficiency. This is illustrated in Figure 11. Recently we have found that the aminonucleoside derived from puromycin, when injected into rats fed a fat-free diet, induces early deficiency of essential fatty acids. This drug induced a hypercholesterolemia of 450 mg./100 ml. and deficiency symptoms within two to three weeks. These results suggest that (1) hypercholesterolemia depletes the reserve of essential fatty acids in the animal faster than a fat-free diet

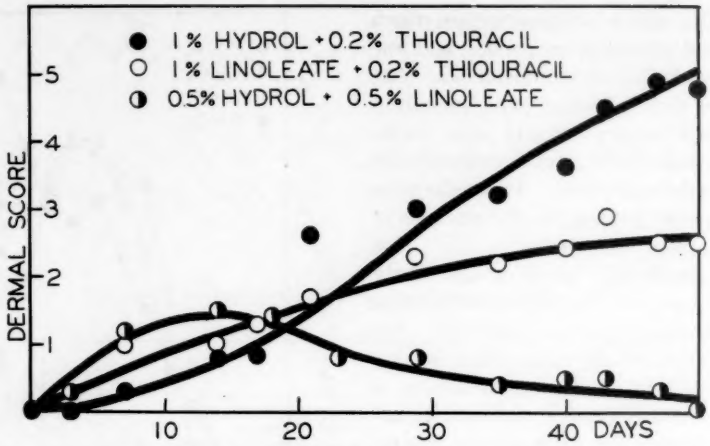


FIG. 11. The effect of hypothyroidism upon dermal symptoms of essential fatty acid deficiency. From: HOLMAN, R. T. and PEIFER, J. J. Chemistry and metabolism of essential fatty acids. *Ann. Rep. Hormel Inst.*, p. 38, 1955–1956.[18]

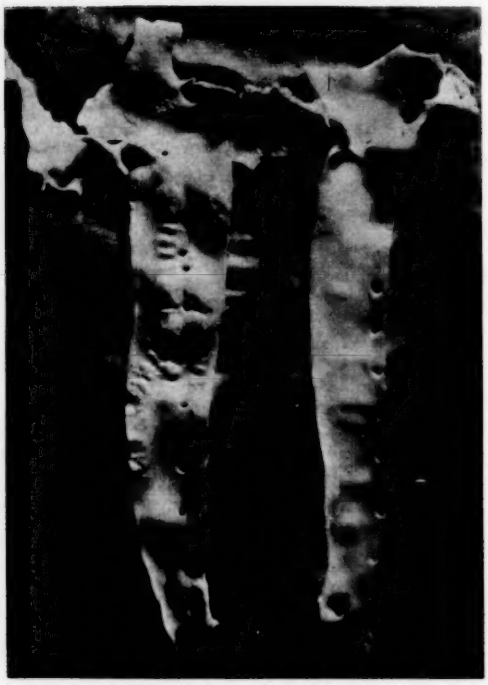


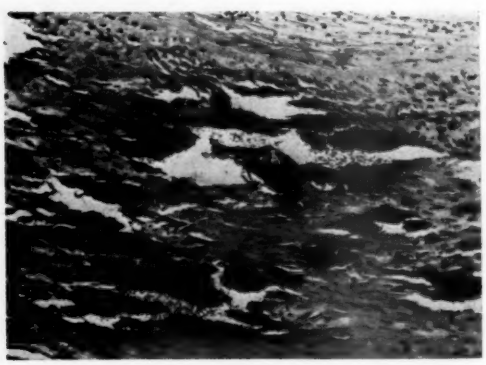
FIG. 12. Lesions of the aorta induced in swine deficient in essential fatty acids. Figures 12 and 13 from HILL, E. G., WARMANEN, E. L., HAYES, H. and HOLMAN, R. T. Effects of essential fatty acid deficiency in young swine. *Proc. Soc. Exper. Biol. & Med.*, 95: 274, 1957.[24]

FIG. 13. Medial calcification in aorta of swine deficient in essential fatty acids. →

and (2) that essential fatty acids are required for the transport of cholesterol.

The essential fatty acids have been shown by Kramar and Levine[23] to maintain capillary resistance. These investigators showed that the capillaries of rats deficient in essential fatty acids ruptured at much smaller negative pressures than did the capillaries of normal rats or rats fed linoleate. Thus, if atherosclerotic lesions are initiated by hemorrhages, a relative essential fatty acid deficiency could increase susceptibility to the disease.

In some recent experiments at our institute, Hill et al.[24] found that in three of six young swine fed an essential fatty acid-free diet lesions of the aorta developed within fifty days. In two swine fed linoleate, and on an otherwise



similar regimen, the lesions did not develop. Histologic examination of the aorta by Dr. Jesse Edwards of the Mayo Foundation revealed the lesions to be medial calcification. The lesions which developed in the first case observed are shown in Figures 12 and 13. Although the lesion is not an intimal lesion, and was not found in all experimental animals, this limited experiment also indicates that essential fatty acids function in the maintenance of a normal arterial wall.

CONCLUSION

This review, representing studies of various lipids and methods of attack, shows that many lipids are involved in atherosclerosis. Essential fatty acids are in some way related to most lipids and the evidence presented seems to indicate that these acids might be at the heart of the problem. It may be trite to say so, but perhaps restoration of lipid homeostasis in man is the problem we should attempt to solve in combating the disease. The problem of atherosclerosis involves the proper balance between dietary essential fatty acids, non-essential fat, fat-soluble vitamins and other nutrients. It is hoped that this hypothesis is provocative enough to stimulate more precise investigation of the relationships of lipids to atherosclerosis using newer, more incisive methodology.

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Dietary Fat and Plasma Cholesterol

A Speculative Review

PETER D. KLEIN, PH.D.*

THE relationships between plasma cholesterol and composition of dietary fat have been widely studied in recent years; it is manifestly impossible to do more than outline the major findings in this review. Furthermore, such an attempt would be repetitious in light of the excellent reviews of the field which are available.[1-3] Therefore, our attention will be confined to the clear-cut phenomena which have emerged and an endeavor will be made to reconcile these with possible mechanisms by which they occur.

It is perhaps a truism to point out that our advances in understanding the regulation of lipid metabolism depend in large part on the methods available for the study of such processes. In general, the more refined our methods the more we are able to distinguish and understand; unfortunately, the availability of such procedures as silicic acid chromatography for separating classes of lipids[4] and of micro alkali-isomerization[5] and gas-liquid chromatography[6] for fatty acid determinations, does not in itself mean that they are readily usable in the clinic. Their adaptation for multiple analyses is a difficult and often treacherous task involving the closest possible liaison between biochemist and clinician.

Aside from the analytic difficulties, the study of this area has been handicapped by the restricted occurrence of the subject material, i.e., the seemingly unique tendency of man to develop atherosclerotic lesions. Attempts to induce similar conditions in experimental animals which may or may not have the same pathways of metabolism do not appear to have resulted so far in a clear-cut reproduction of the syndrome. On the other hand, the conduct of studies in man is usually expensive, laborious, and time-consuming in terms of numbers of individual cases. Moreover, it appears that this situation is unlikely to improve, but will in fact become worse as we find that more extensive as well as more specific information is required about these processes in man.

From the information which is presently available I shall discuss three points: (1) the relationships which have been observed between the polyunsaturated fatty acids and plasma cholesterol; (2) the possible functions of plasma cholesterol esters; and (3) the mechanisms whereby polyunsaturated fatty acids might bring about changes in the level of circulating cholesterol.

DIETARY LIPID: PLASMA CHOLESTEROL RELATIONSHIPS

About seven years ago, Kinsell and coworkers in this country[7-9] and Groen and his colleagues[10] in Europe pointed out that vegetable fats differed from animal fats in their effect on plasma cholesterol, the former lowering these levels. Since then, this phenomenon has been intensively studied; the findings of these workers as well as those of Ahrens,[11] Beveridge[12] and others are summarized in the following points:

(1) The addition of saturated fat to the diet raises the level of plasma cholesterol.

(2) Reducing the dietary content of saturated fat will lower the cholesterol levels, but

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the addition of an unsaturated oil will produce a further decrease.

(3) Isocaloric substitution of an unsaturated fat (predominantly linoleate triglycerides) for a saturated fat will produce these same changes even though the total level of fat in the diet is high.

(4) On the basis of the composition of the oils, the active ingredient appears to be the polyunsaturated fatty acids. This has been essentially substantiated by producing the same effects with ethyl linoleate. On the other hand, the reduction of plasma cholesterol levels in patients fed menhaden oil[13] suggests that this action is independent of the structural configuration required for "essential fatty acid" activity in the rat. The fatty acids of menhaden oil are inactive when tested for their ability to alleviate a deficiency of essential fatty acids in rats.

(5) The major portion of the change brought about by unsaturated fat in the diet takes place within three to eight days, although a slow decrease continues for some time.

(6) The cholesterol esters of plasma tend to reflect the composition of the diet in that the substitution of an unsaturated fat in the diet raises the per cent of unsaturated esters in the plasma, even though the total level of the cholesterol is decreased.[14] Conversely, a rising plasma cholesterol level on a diet in which the fat is predominantly saturated is accompanied by an increase in the saturated esters.

The basic relationship which emerges from these findings may be described as follows. With a constant level of fat in the diet, alterations in the composition of the fat (i.e., changes in the per cent saturated, mono- and polyunsaturated fatty acids) will bring about changes in the size of the plasma cholesterol pool. For the sake of the subsequent discussion on possible mechanisms it should be pointed out that the *direction* of such changes is a function of the species in which it is studied. Thus, in rats[15,16] and calves[17] increases in the unsaturated fat component will result in increased plasma cholesterol levels, while in mice[18] and dogs,[19] as in man, this treatment promotes a reduction. Although any mechanism which we may postulate is not rigorously obligated to account for these differences, some reconciliation would seem advantageous.

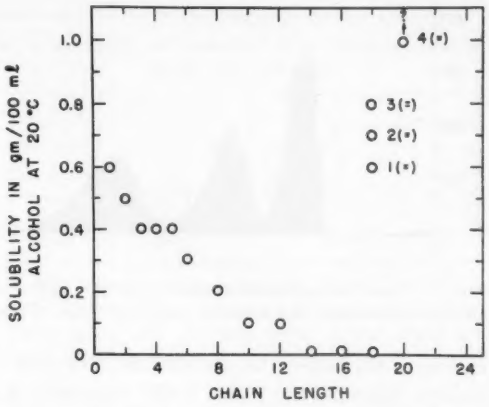

FIG. 1. Solubilities of cholesterol esters in alcohol. Data taken from Page and Rudy.[25] The point for arachidonate was not reported and hence is estimated from structural considerations.

POSSIBLE FUNCTIONS OF PLASMA CHOLESTEROL ESTERS

Most speculations on the function of plasma cholesterol esters fall into one of three categories: (1) transport of cholesterol; (2) transport of fatty acids; and (3) modification of blood lipid solubility.

With respect to the first point, the transport of cholesterol by plasma esters, there are several objections which would have to be overcome before such a concept could be accepted. It has been shown that in rats the liver is the chief if not the sole source of plasma esters.[20,21] Furthermore, the removal of cholesterol from plasma is also mediated almost exclusively by the liver.[22] The establishment of a system involving the net transport of cholesterol via the plasma esters thus seems difficult.

On the other hand, the transport of fatty acid by this means is not excluded. Miller, Roheim and Spitzer[23] have reported that perfusion of the rabbit's heart with hypercholesteremic serum resulted in a disappearance of ester fatty acid, even though the total cholesterol remained unchanged. While it has been shown by Lipsky et al.[24] that the turnover rate of the saturated fatty acids of plasma esters is too slow to account for any sizeable

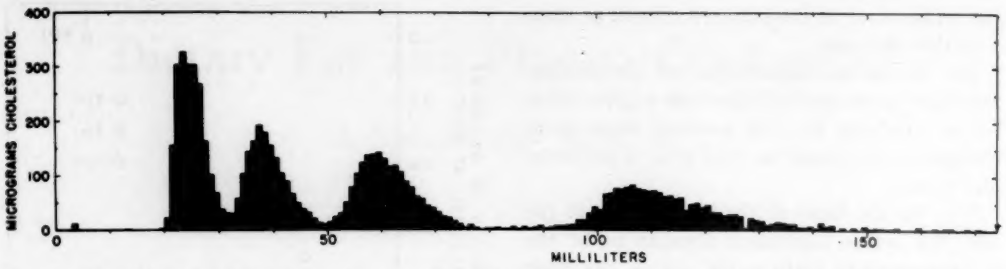

FIG. 2. Separation of model cholesterol esters. The order of emergence is cholesterol palmitate, cholesterol oleate, cholesterol linoleate and cholesterol arachidonate. Total load, 9.21 mg. of ester as cholesterol.

caloric contribution to metabolism, the possibility remains that this could represent a specialized pathway for the transport of polyunsaturated fatty acids.

The ability of the cholesterol esters to modify the solubility of other blood lipids is best appreciated on the basis of Figure 1, which shows the solubility of the esters in alcohol as a function of chain length and unsaturation. The marked differences that exist between saturated and unsaturated esters suggest that in an aggregate such as a lipoprotein, the nature of the fatty acid moieties in the esters could profoundly influence the ability of the aggregate to bind other lipids. For example, the greater the mean unsaturation of these esters, the greater will be the polarity of the mixture and hence the greater the miscibility with triglycerides and other lipids. Along this line, further studies on the physical structure of the lipoproteins would be of great value in assessing this possibility.

POSSIBLE MECHANISMS WHEREBY POLYUNSATURATED FATTY ACIDS MAY ALTER PLASMA CHOLESTEROL

In considering the possible mechanisms whereby dietary fat can influence the level of plasma cholesterol, our first instinct is to ask: in what stages of cholesterol metabolism do fatty acids *per se* participate? Two such stages are evident: acetate biogenesis and ester formation. Beyond such stages, we are forced into postulating relationships that involve the biochemical equivalent of action-at-a-distance (except, of course, the process of cholesterol absorption which will not be considered here); such relationships may indeed exist but their mechanisms are too complex to analyze directly. Under such relationships we might include the studies of Gordon et al.[26] on bile-acid excretion as influenced by the character of dietary fat. These studies and those of Hellman and associates[27] will be discussed presently.

With regard to acetate biogenesis, we have little or no information as to possible differences in rate between individual fatty acids *in vivo*. Ahrens[28] has referred to unpublished work indicating that cholesterol biosynthesis is unaffected by the character of the dietary fat. Similarly, Alfin-Slater and co-workers[29] reported that the amount of newly synthesized cholesterol is the same in livers of animals fed a low fat or high fat diet. On an over-all basis, it is difficult to postulate where such a difference might arise other than in the initial stages of fatty acid degradation, the formation of the acyl-CoA derivative.

Likewise, we can say little at this time as to specific mechanisms whereby dietary fat could influence cholesterol degradation and/or excretion. Gordon[26] has reported that feeding patients hydrogenated coconut oil gave rise to increased serum cholesterol but did not alter bile-acid excretion, whereas feeding sunflower oil promoted both a drop in the serum cholesterol level and an increased excretion of bile acids. In a subsequent paper, Lewis[30] has suggested that vegetable oils accelerate the degradation of cholesterol to bile acids. On the other hand, Hellman et al.[27] found reciprocal changes in the fecal sterol excretion with changes in plasma cholesterol levels; i.e., butter caused a decreased excretion of sterols while corn oil led to an increase. Still another viewpoint is that of Kinsell and co-workers[31] who

have reported that no consistent changes in either direction result from changes in the fat composition of the diet.

COMMENTS

Further discussion would seem to depend upon the establishment of two points: (1) the true reciprocality of the rate of excretion of cholesterol and its end products with the plasma cholesterol levels; and (2) whether this represents a true change in the rate of excretion or a transient readjustment of pool size. The latter point is one of considerable concern from the standpoint of possible mechanism. The postulation of changes in the basic rate of metabolism of cholesterol (herein considered in terms of the flux of cholesterol through the organism, i.e., milligrams per hour) suffers from one major drawback: its failure to explain why the decline in plasma cholesterol ceases when a lower level is reached instead of continuing as long as the oil is fed. If there were simply a net difference between the rates of synthesis and degradation or excretion, such a decline should continue indefinitely; it obviously does not; hence one must then postulate additional circumstances which limit these changes. These discrepancies are avoided in the alternative possibility: a change in the pool size of the circulating cholesterol by mechanisms not necessarily dependent on the actual rates of biosynthesis and catabolism of cholesterol.

One of the most promising of such mechanisms has seemed to us to be the possibility that the turnover rate of cholesterol in esterified form could differ according to the fatty acid to which it is esterified. In other words, the rate at which cholesterol could be transported as, for example, cholesterol palmitate might differ appreciably from the rate at which the linoleate ester is transported. Under such circumstances, the level of plasma cholesterol could conceivably be a function of the individual turnover rates and hence, of the composition of the esters. Changes in the dietary fat, as has been pointed out, are reflected in the composition of the plasma esters. The cholesterol levels might then inversely reflect the turnover rates of the predominant esters, dropping as the joint rate increased and rising as the rate decreased. Under such conditions the rate of excretion of cholesterol and its metabolites would remain relatively constant, fluctuating upward only as the superfluous segment of the plasma pool was discarded or downward during the time the pool was being expanded. In both cases, however, after such changes the rate would be expected to return to a characteristic level and would not differ according to the type of dietary fat.

TABLE I
Reproducibility of Cholesterol Ester Fractionation on Silicic Acid Columns*

Fraction	I	II	III	Average†
Saturated	7.7	6.2	5.0	6.3(0.8)
Oleate	23.0	23.4	20.2	22.2(1.0)
Linoleate	60.4	57.7	62.1	60.0(1.9)
Arachidonate	7.4	11.8	12.9	10.7(1.7)

NOTE: All figures are percentages.
* Sample: Human plasma cholesterol esters.
† Including the standard error of the mean (in parentheses).

The study of the possibility of heterogeneity in cholesterol ester renewal has been under way in our laboratory for several years; some of the findings are of interest to consider here. Initially, we sought a method for the isolation of individual esters in an intact condition. Although studies of ester composition are possible by means of conventional technics involving hydrolysis and identification of the fatty acid population, the establishment of differences in ester turnover rates requires the ability to identify a given cholesterol molecule in terms of the fatty acid to which it was esterified. Such a method appears to be provided in the silicic acid chromatography procedure recently described by Klein and Janssen.[32] By suitable modification of the procedures of Barron and Hanahan[33] resolution of four components, esters of saturated, oleic, linoleic and arachidonic acids, has been achieved. The separation of model compounds is illustrated in Figure 2, and the application to biological mixtures is shown in Figure 3. Recoveries of individual esters average 91 per cent and total recoveries are generally 95 per cent or better. The reproducibility of the method, in-

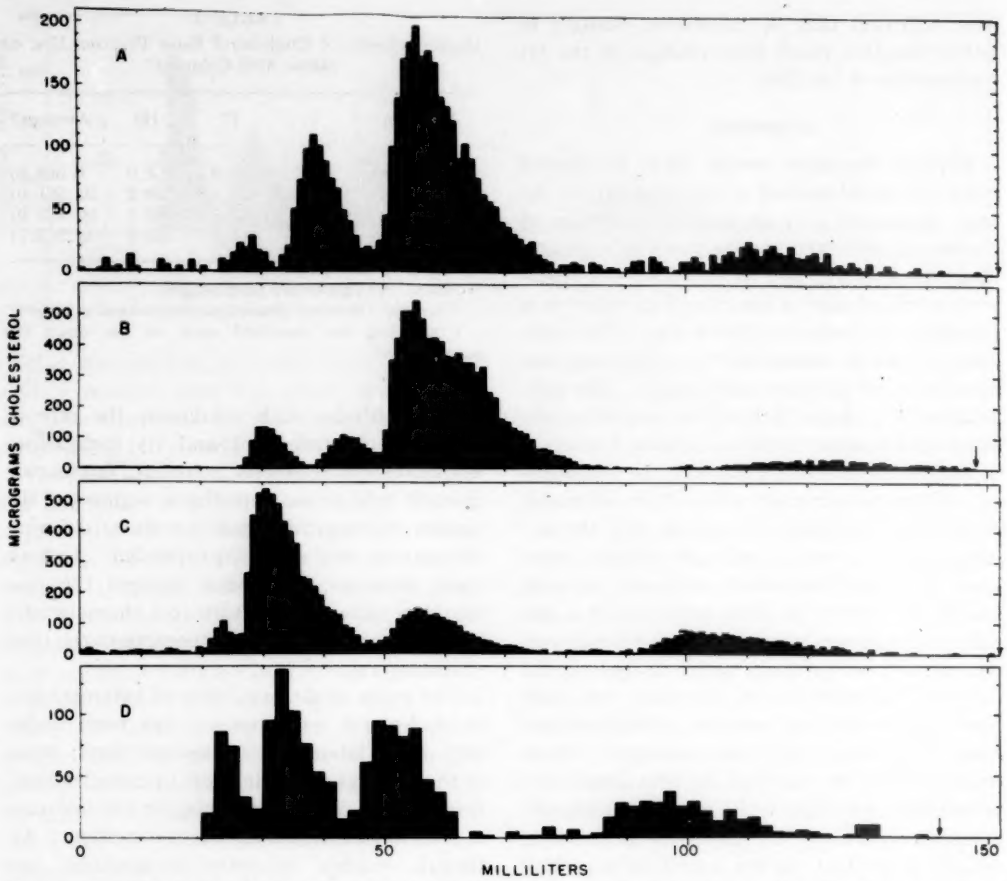

FIG. 3. Separation of cholesterol esters in biological mixtures. A, esters of human plasma. B, esters of beef plasma. C, esters of rat plasma. D, esters of rat liver.

dicated by the values in Table I is 3 per cent of the value for components comprising 20 per cent or more of the total and increases to 16 per cent when the component is less than 10 per cent.

Using this method we have studied the incorporation of newly synthesized cholesterol into each of these ester categories of liver of the rat at one-half hour and four hours after the injection of labeled acetate.[34] The data obtained are shown in Table II, which lists the ester categories, the number of samples taken from within each peak and the specific activities of the digitonides derived from the esters with their associated standard error. These values were subjected to a one-way analysis of variance and the probability of homogeneity was calculated. The likelihood that these specific activities, at one-half hour after the injection of activity, were drawn from a homogeneous population was less than 0.05, i.e., the individual categories were indeed of different specific activities. It was of interest to relate the specific activities of these digitonides to those of the cholesterol derived from cholesterol oleate; these values are shown in Table III. In each of the runs the order of decreasing relative specific activity was found to be the same: oleate, linoleate, saturated and arachidonate esters.

On the other hand, after four hours, the probability of homogeneity had increased to

TABLE II

Specific Activities of Cholesterol Digitonides from Various Fractions of Liver Cholesterol Esters Thirty Minutes after Injection of Sodium Acetate-1-C^{14}

Ester Fraction*	Per Cent Total Ester	Mean Specific Activity (disintegrations per min./mg.)	Standard Error	Probability of Homogeneity (p)
Run 12: Single Rat Liver				
Saturated (3)	7.8	3,912	995	<0.001
Oleate (5)	35.1	4,784	201	
Linoleate (7)	34.1	4,291	188	
Arachidonate (9)	23.1	2,339	212	
Run 21: Two Pooled Rat Livers				
Saturated (3)	12.9	1,149	292	$0.05 > p > 0.01$
Oleate (7)	31.7	2,403	305	
Linoleate (6)	31.6	1,833	218	
Arachidonate (3)	23.8	1,158	155	
Run 25: Two Pooled Rat Livers				
Saturated (4)	12.2	4,889	529	<0.001
Oleate (8)	39.6	8,001	495	
Linoleate (8)	29.9	6,019	350	
Arachidonate (4)	18.2	967	112	

* Numbers in parentheses are the number of samples taken in fraction for specific activity measurement.

0.2 and the relative specific activities were quite similar, indicating that comparable portions of the esters had been replaced (Table IV). This determination was necessary to establish that the differences in incorporation rate observed were not the result of differences in the proportion of each ester undergoing replacement.

These data indicate that it is likely that these esters are indeed replaced at differing rates and thus they lend credibility to the hypotheses outlined previously. Nevertheless, there remains the need to study these phenomena in plasma, since the secretion of cholesterol esters into the circulation represents another point at which diversity could exist. These studies are presently under way. It is of interest to note, however, that in the rat increased levels of plasma cholesterol are associated with high levels of arachidonic acid in the esters,[15] which is at least superficially in agreement with the finding that this ester has the lowest relative turnover rate in the liver of the rat.

While it is heartening to have logic confirmed by fact, the speculative nature of this entire discussion should be emphasized. Much work remains to be done before any sizeable number of these points can be considered to be established conclusively; if and when they are, their relationship to the multitude of other factors participating in cholesterol and lipid metabolism must be studied. It is unlikely that the phenomena discussed earlier can be completely

TABLE III

Specific Activities of Cholesterol Esters Relative to Cholesterol Oleate Thirty Minutes after Injection of Sodium Acetate-1-C^{14}

Source of Cholesterol	Run 12	Run 21	Run 25
Cholesterol oleate	1.00	1.00	1.00
Cholesterol linoleate	0.90	0.76	0.75
Cholesterol "palmitate"	0.82	0.48	0.61
Cholesterol arachidonate	0.49	0.48	0.12

TABLE IV

Specific Activities of Cholesterol Digitonides from Various Fractions of Liver Cholesterol Esters Four Hours after the Injection of Sodium Acetate-1-C^{14}

Ester Fraction*	Mean Specific Activity (disintegrations per min./mg.)	Standard Error	Probability of Homogeneity (p)	Specific Activity Relative to Oleate
Run 32: Two Pooled Rat Livers				
Saturated (8)	1,201	65	0.2	0.88
Oleate (9)	1,359	112		1.00
Linoleate (13)	1,236	72		0.91
Arachidonate (6)	1,465	138		1.08

* See footnote to Table II.

explained by such mechanisms as heterogeneity of ester renewal, but the introduction of these new parameters may serve to increase our understanding of this metabolic area.

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Editorial

Sequential Analysis in Nutrition Studies

In conducting nutrition surveys and various kinds of experimental work, there is a need for a relatively rapid method of evaluating the data as they are collected. Such a method would not only prevent wasted effort in collecting data after sufficient information is available to make a decision but would also prevent the termination of the work before enough was known to reach a definite conclusion. It would be extremely convenient to have the various hypotheses to be tested stated in advance and to be able to evaluate the results as they are collected. As soon as a decision was reached with regard to any particular hypothesis, within probability limits previously determined, the collection of such data could be stopped and additional effort expended in other more profitable directions.

In a survey, for example, blood samples might be collected for several determinations including hemoglobin and cholesterol. The hypothesis to be tested might be that the average hemoglobin value lies between 14 and 15 gm. per 100 ml. and that the mean serum cholesterol value is between 200 and 240 mg. per 100 ml. There is no reason to suspect that the same number of samples will be required to permit a judgment on each of the questions asked. In areas in which the population is relatively anemic, only a few samples would be required to determine whether or not the hypothesis with regard to hemoglobin was correct and the determination of hemoglobin could be dispensed with or an alternate hypothesis proposed. In other areas a decision with regard to the serum cholesterol level might be made with few samples, but many more might be required to reach a decision regarding the hemoglobin level.

Sequential analysis might well offer possibilities in this area. The technics involved were developed during World War II and were used to increase the efficiency of the inspection of products. The purpose of this paper is to describe briefly the situations in which sequential procedures might be applied and, through the use of an example, to provide a general understanding of the method and its potential value in the nutrition field.

Several recent texts on statistics have chapters devoted to the subject. The simplest description we have found, including an example similar to many of those of interest to nutritionists, appears in the text of Rosander.[1] The example is on page 659 and reference to this will make the development of our example clearer. The reader who is interested in the theoretical basis, development and limitations of the technic is cautiously referred to Wald's "Sequential Analysis,"[2] the standard book in this field. The caution is due to the rather overwhelming mathematics involved. Very likely, the average person will be content with a general understanding of the method and its usefulness and will consult better qualified people to be assured he does not go astray in application.

Sequential procedures have been developed to test most of the hypotheses generally found in nutrition studies. In surveys, for example, they make it possible to decide whether the proportion of "successes" (however defined) in a sample differs significantly from an expected proportion or whether the average value of a measurement is significantly higher or lower than a so-called "normal" value. In experimental studies in which paired observations are used, differences between proportions or between mean values can be readily tested for significance.

Although various types of questions can be answered through the use of sequential

analysis, the actual test procedure is quite similar from situation to situation. In each case appropriate observations are made one by one and their sum analyzed consecutively as each new observation is made. It is this approach that makes the sequential procedures differ from classic tests, such as the t test, and that, quite often, makes it possible to reach a decision with far fewer observations than would otherwise be used. There are other common elements in all sequential tests. What they are is probably best explained through an example.

The example we would like to discuss has to do with deciding whether the mean value in a sample differs significantly from an expected or "normal" value. Evidence[3] indicates that adult men in the United States have an average serum cholesterol value of approximately 240 mg. per 100 ml. with a standard deviation of approximately 48. The standard deviation of other populations with quite different mean cholesterol values appears to be of the same order of magnitude.[4] This is fortunate since this type of sequential test assumes that the standard deviation is known in advance. In this example, cholesterol determinations for four groups will be used, namely, a sample of Ethiopian adults, of preparatory school boys between fourteen and eighteen years of age, a random group of adult men, and a group from a cardiac clinic who might be suspected of hypercholesterolemia. For each group, the null hypothesis is that the mean does not differ significantly from the expected mean of 240 mg. per 100 ml. Before testing begins, the experimenter must decide on practical grounds how much smaller or larger the sample mean must be to indicate a real difference. For example, a mean of 239 is certainly less than 240, but the difference is so small that it would probably not be considered an important one to detect. Let us decide for our example that the means must differ by at least 25 mg. per 100 ml. from 240 in order to be of practical importance. With this range of acceptable values specified, we then test whether the mean in question is at least as small as 215 or as large as 265.

In sequential analysis, as in all significance testing, there is a possibility of drawing the wrong conclusion from the sample results. To control this potential error, the experimenter must specify in advance the risk of being wrong that he is willing to tolerate. There are actually two ways of wrongly rejecting the null hypothesis in our example. First, we may conclude the mean is as large as 265 when, in fact, it is as small as 215. This risk of doing this is called alpha. Secondly, we may conclude the mean is as small as 215 when it is really as large as 265. This risk is called beta. For our example, we will specify that both alpha and beta should be 2 per cent. That is, we are willing to be wrong two times out of 100 in rejecting the null hypothesis either above or below our specified range.

With this much information available, it is possible to construct boundary lines which will specify for each sample size how large (or small) the sum of the cholesterol values must be to conclude that the mean of the group involved is as large as 265 (or as small as 215).

As given in the example of Rosander,[1] the formulas for these lines, in an example like this, are:

$$y_1 = \Sigma x = \frac{-bs^2}{\bar{x}_2 - \bar{x}_1} + \left(\frac{\bar{x}_1 + \bar{x}_2}{2}\right) n$$

$$y_2 = \Sigma x = \frac{as^2}{\bar{x}_2 - \bar{x}_1} + \left(\frac{\bar{x}_1 + \bar{x}_2}{2}\right) n$$

where $\bar{x}_1$ and $\bar{x}_2$ are the lower and upper means of interest, namely 215 and 265

s = Expected standard deviation, namely 48

n = Number of observations

$a = \log_e \frac{1-\beta}{\alpha}$ α: Risk of wrongly accepting $\bar{x}_2$, namely, 0.02

$b = \log_e \frac{1-\alpha}{\beta}$ β: Risk of wrongly rejecting $\bar{x}_2$, namely, 0.02

For our example, the lines, which are plotted in Figure 1, become:

$$y_1 = \Sigma x = -176.2 + 240n$$

$$y_2 = \Sigma x = +176.2 + 240n$$

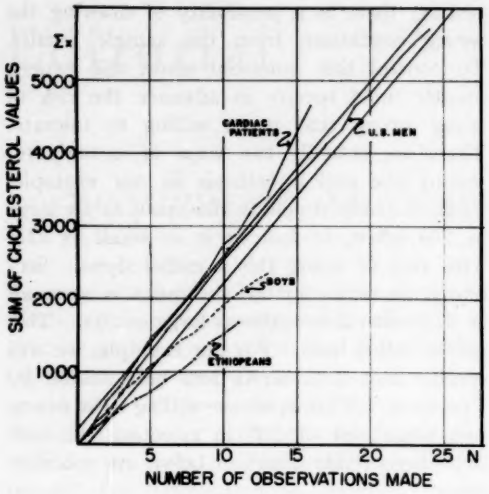

FIG. 1. The test chart for the evaluation of average serum cholesterol values.

These have been plotted in Figure 1, the points being obtained by substituting various values for n in the equations.

It is worth a comment here that these lines do not depend in any way on observations from the four samples under investigation. This means that they can be used by anyone interested in comparing the cholesterol level of a particular group with the average value for adult American males, provided, of course, they are willing to accept 215 and 265 as reasonable dividing values and are willing to be wrong 2 per cent of the time in either direction.

The test procedure itself is a simple one. As each observation is made, i.e., as each cholesterol level is determined, it is added to the preceding ones and their sum is plotted on Figure 1. As long as the sum remains between the boundary lines, observations continue to be made. As soon as the sum crosses a line, either above or below, testing stops and the appropriate conclusion is drawn.

For each of our four groups, successive values as they were determined and the cumulative sums are given in Table I. The sample mean values are included for information but not used in the test. The sums are plotted in Figure 1.

For the Ethiopians, the sum of the values rapidly falls below the limits established by the hypothesis, indicating that their average cholesterol level is significantly lower than that of American males and is at least as small as 215. In fact, only three determinations needed to be made in this group since their sum at that point fell below the boundary line. For illustrative reasons only, a few more observations were made and their sum recorded to show that the difference becomes more striking as the sample size increases.

The prep-school boys also have an average cholesterol level that is significantly low, but it was necessary to observe six or seven boys before being able to draw this conclusion.

The cardiac patients can be defined as hypercholesterolemic, above 265 mg. per 100 ml. by the tenth observation although only after the fourteenth is this unequivocal.

Finally, the random sample of adult males shows no tendency to depart from the boundary lines. This is to be expected since they are a sample from the population with which they are being compared. In a situation like this, namely the sum remaining within the lines as the sample size increases indefinitely, there are methods available to determine the maximum number of observations that need to be made.

Other sequential tests differ from the one illustrated in the actual statistics on which the test is made and in the formulas for the boundary lines, but all are based on the use of such boundary lines. They have in common the need for specifying a range of acceptable values, such as the range from 215 to 265 mg. per 100 ml., and for specifying the risk that will be tolerated for being wrong in either direction, such as α and β both 0.02. Most of the methods of sequential analysis require a reasonable estimate of the underlying standard deviation and, in addition, assume that the distribution involved is essentially normal.

Before extensive use of the method can be made, it will be necessary to know more about the nature of the distributions encountered with various tests, and under varying conditions, and the degree to which errors in the estimated standard deviations influence the

TABLE I
Serum Cholesterol Data from Four Samples Tested by Sequential Analysis

Sample No.	Ethiopians		Boys		Adult Men		Cardiac Patients	
	Serum Cholesterol (mg. %)	Σx	Serum Cholesterol (mg. %)	Σx	Serum Cholesterol (mg. %)	Σx	Serum Cholesterol (mg. %)	Σx
1	227	227	187	187	255	255	258	258
2	103	330	196	383	189	444	338	596
3	163	493	345	728	272	716	249	845
4	134	627	145	873	308	1,024	298	1,143
5	93	720	199	1,072	248	1,272	307	1,450
6	153	873	157	1,229	279	1,551	193	1,643
7	129	1,002	179	1,408	230	1,781	236	1,879
8	163	1,165	126	1,534	238	2,019	248	2,127
9	227	1,392	203	1,737	267	2,286	275	2,402
10	122	1,514	185	1,922	269	2,555	355	2,757
11	155	1,669	171	2,093	222	2,777	214	2,971
12	...	...	137	2,230	206	2,983	279	3,250
13	...	...	122	2,352	254	3,237	179	3,429
14	...	...	...	...	247	3,484	428	3,857
15	...	...	...	...	183	3,667	424	4,281
16	...	...	...	...	228	3,895	389	4,670
17	...	...	...	...	230	4,125	...	...
18	...	...	...	...	187	4,312	...	...
19	...	...	...	...	340	4,652	...	...
20	...	...	...	...	160	4,812	...	...
21	...	...	...	...	246	5,058	...	...
22	...	...	...	...	245	5,303	...	...
23	...	...	...	...	195	5,498	...	...
24	...	...	...	...	193	5,691	...	...
Mean	151.7		180.9		237.1		291.9	

applicability of the test. Too little attention has been paid in the past to this statistical parameter. It is clear that the data are often markedly skewed[5] and some transformation may be needed before the data can be properly treated. However, in many laboratories data, which have been collected in the past under varying conditions, are now available. These might be used for retrospective tests of the method and current surveys could provide tests in the field. Preference for any method, statistical or otherwise, must depend upon its performance under actual usage.

Finally, an advantage of sequential analysis might be that it requires the formulation of the hypotheses to be tested in rather exact form prior to the collection of the data. Most nutrition surveys to date have been performed with only general questions in mind and a "let's see what happens" attitude. This is often useful and necessary. In many other situations, however, particularly in which standard methods are applied, we should have reached the degree of maturity where we know what we want to know, the accuracy with which we wish to make the decision, and desire an answer with a minimum expenditure of time and energy. It is often true that the quality of the answer obtained depends upon the question asked.

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Nutrition News

First Annual Congress of the Nutrition Society of Southern Africa

The Nutrition Society of Southern Africa held its first Annual Congress in Pretoria on November 16 and 17, 1959. Symposia were held on Proteins in Nutrition, with a discussion of such subjects as kwashiorkor, assessment of nutritive value and relation to atherosclerosis.

American Board of Nutrition

The American Board of Nutrition will hold the next examinations for certification as a Specialist in Human Nutrition during the week of April 11–15, 1960, in Chicago, Illinois. Candidates who wish to be considered for these examinations should forward applications to the Secretary's office not later than March 1. Application forms may be obtained from the Secretary, Dr. Robert E. Shank, Department of Preventive Medicine, Washington University School of Medicine, Euclid and Kingshighway, St. Louis, Missouri.

Fifth International Congress on Nutrition

The Fifth International Congress on Nutrition, organized by the American Institute of Nutrition and the U.S.A. National Committee for Nutritional Sciences of the National Academy of Sciences, will be held in Washington, D. C., September 1 to 7, 1960, under the auspices of the International Union of Nutritional Sciences.

Dr. C. Glen King, Executive Director of The Nutrition Foundation, New York City, has been named President of the Congress; Dr. Elmer V. McCollum, Professor Emeritus of Biochemistry, Johns Hopkins University, is Honorary President. Dr. Paul György, Professor of Pediatrics, University of Pennsylvania, Philadelphia, is Chairman of the Organizing Committee.

Eminent nutrition scientists from all over the world will participate in the Congress. In addition to papers submitted for the usual scientific sessions, a number of panel discussions and symposia on major problems in the field of nutrition in its broader aspects will be presented.

There will also be scientific and industrial exhibits. Visits to various scientific laboratories and other places of interest will be arranged. A special program for women guests and general entertainment for all registrants are being arranged.

For additional information write to Dr. Milton O. Lee, General Secretary, 9650 Wisconsin Avenue, Washington 14, D. C.

Sixth International Congress for Internal Medicine

The Sixth International Congress for Internal Medicine will be held in Basel, Switzerland, August 24–27, 1960. This Congress will be organized in conjunction with the Swiss Society for Internal Medicine. For further details apply to the Secretariat of the Sixth International Congress for Internal Medicine, 13, Steinentorstr., Basel.

Reviews of Recent Books

Ciba Foundation Symposium on Carcinogenesis. Mechanisms of Action, edited by G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown & Company, Boston, 1959, pp. 336, $9.50.

This volume is dedicated to the memory of the late Sir Ernest Kennaway and contains papers arranged in relation to the Seventh International Cancer Congress in London. Professor A. Haddow was Chairman of the Symposium.

The scope of the papers may be indicated by a partial list of the topics: mechanism of carcinogenesis of viruses, hormones, pharmacologic approach, ionizing radiation, protein binding, metals and metal chelation, etc.

Sir Macfarlane Burnet's able summary stresses that there is no one basic mechanism, but "... a variety of ways in which chemicals, radiations, viruses and hormones can influence cellular and internal environmental processes so as to release clones of cells into abnormal and destructive proliferative activity." E. COHEN

International Conference on the Influence of Living and Working Conditions on Health, Cannes, September 1957, edited and published by the International Medical Association for the Study of Living Conditions and Health, Vienna, 1959, pp. 607, $6.00.

This is a compilation of the many long and short reports presented at the conference noted in the aforementioned title. Articles appear in English, French, Russian, German and Spanish with summaries translated into all these languages. Many subjects are covered, from the effect of fatigue on the health of industrial workers to ionizing radiation and heredity.

Special attention is paid to nutrition. The conference, with representation from thirty nations, concluded that malnutrition is the world's most common disease. They recognized the need for a wide effort to promote social and economic development of the various populations in order that all may obtain adequate foodstuffs for a balanced diet. As Dr. J. DeCastro says in his summary of the nutritional papers, "We should never forget that the nutritional problem must be dealt with within the framework of regional economics."

There are certainly many interesting papers presenting uncommon views on topics which are inadequately considered today. For this reason and because the viewpoints of our colleagues in other countries are always significant, this conference report can be recommended. S. O. W.

The Mast Cells, by James F. Riley. E. & S. Livingstone, Edinburgh, 1959 (Williams & Wilkins, exclusive American agents), pp. 182, $6.75.

This monograph is divided into two parts, the first dealing with the literature of mast cells. The second part is essentially a summary of the author's experiments concerning the function of mast cells and carried out primarily in cattle and rodents. They are based on observations of mastocytosis occurring in the skin of mice painted with carcinogens and of the prolonged coagulability of blood in shock, and aim to show that histamine release may play a major part in the function of mast cells. Histamine liberators, e.g., stilbamidine, anaphylatoxin or compound 48/80 were found to cause swelling, degranulation and disruption of mast cells, while some antihistamines counteracted these effects. There are differences in structure and composition of mast cells even in such closely related species as rat and mouse as evidenced by their sensibility to histamine liberators. On the other hand, not all histamine present in the body is produced by mast cells, nor do all mast cells react to histamine liberators. The author plans to trace the source of the large amount of 5-hydroxytryptamine seen in conditions associated with mastocytosis.

This brochure makes interesting reading and is a useful source of information regarding nature and function of mast cells, about which there is still much to learn, as pointed out in the foreword by Sir Henry H. Dale. The book should be welcomed by everyone who is planning to carry out original investigations along these lines. It is far from being one-sided, points to many problems without dogmaticism and still is concise and well written. An excellent bibliography concludes the book. M. SILBERBERG

Ciba Foundation Symposium on the Regulation of Cell Metabolism, edited by G. E. W. Wolstenholme and C. M. O'Connor. Little, Brown & Company, Boston, 1959, pp. 387, $9.50.

This symposium had as its distinguished chairman Sir Hans Krebs, who gave the opening address on "Rate-limiting factors in cell respiration." The other papers were also by outstanding researchers. Regulation by intracellular structure, topographical segregation of controlling enzyme systems and control of respiration by energy-rich intermediates of oxidative phosphorylation were among topics discussed. Quantitative aspects of control of oxygen utilization and a comparative study of current views on probable meta-

bolic pathways of carbohydrate metabolisms were presented, as well as some aspects of hormonal effects on oxidative pathways in the liver and on mitochondria. The significance of the mitochondria for alternative pathways of electron transport was hypothesized. The Pasteur effect in relation to limiting factors in glycolysis of ascites tumor cells was described, emphasizing the importance of the availability of inorganic phosphate in limiting the rate of both lactic acid production and glucose uptake. The role of triphosphopyridine nucleotide in the regulation of glycolysis in cell-free preparations by decreasing glycolysis was reported to be enhanced by addition of liver mitochondria, which can oxidize the reduced form of the nucleotide.

There were papers on yeast cell fermentation and enzyme induction by bacteria that developed hypotheses of feedback mechanisms, or "automatic adjustment." There is an abundance of diagrams, and equations that are necessary for understanding current theories and hypotheses. The general discussion at the end of the symposium contains a considerable amount of information on the use of the word "feedback" by biochemists; the term came originally from purely physical systems. However, perhaps a question can be raised as to whether feedback of the engineer and of the chemist can be directly compared. This reviewer felt that a knowledge of feedback of electronic circuiting might be a better prerequisite to reading portions of the symposium than a background in biochemistry. At any rate, there is no doubt that the book is the first to feature such a variety of aspects of non-hormonal regulation of cellular metabolism. It should be a provocative addition to the bookshelf of all biochemists engaged in fundamental research.

E. COHEN

Essentials of Medicine. The Art and the Science of Medical Nursing, 18th edition, by Charles P. Emerson and Jane S. Bragdon. J. B. Lippincott Co., Philadelphia, 1959, pp. 857, $6.75.

This standard medical text for nurses is now in the eighteenth edition, having first been published fifty-one years ago. The book's success is no doubt due to its comprehensive coverage, clear language and numerous illustrations. The discussion of various disorders is followed by a useful section, "Treatment and Nursing Care." The book can be recommended as an authoritative reference in medical nursing. R. S. W.

Food, The 1959 Yearbook of Agriculture. The U. S. Department of Agriculture, Washington, D. C., 1959, pp. 736, $2.25.

This Yearbook of Agriculture is the latest in the series of annual volumes that have been printed for 110 years. Its sixty-five chapters and 736 pages tell much of what seventy-two nutrition experts in the U. S. Department of Agriculture, other Federal agencies and twelve colleges know about weight control, food energy, vitamins, proteins, amino acids, fats, carbohydrates, calories, minerals, quality in food, costs, fads, habits and nutrition programs.

It has thirty-six pages of simple recipes that further the book's main theme—"for good health, eat some of the basic foods every day." Fourteen pages give questions (with answers) that many people have asked about food. One section spells out the nutritional needs of expectant and nursing mothers, infants, teenagers and young adults and people over twenty-five years of age.

Other chapters discuss grades of meat, eggs and fish; freezing, canning, storing and preparing food at home; food plans at different costs, changes in U. S. diets, learning and teaching good eating habits, school, lunches and our future food supplies and needs.

Representing, as it does, authoritative opinion, the book can be highly recommended as one maintaining the well known level of excellence in previous Yearbooks. S. O. W.

BOOKS RECEIVED

Books received for review by THE AMERICAN JOURNAL OF CLINICAL NUTRITION are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for a more extensive review.

History of the American Dietetic Association, edited by Mary I. Barber, J. B. Lippincott Co., Philadelphia, 1959, pp. 328, $6.00.

Essentials of Medicine: The Art and the Science of Medical Nursing, 18th edition, by Charles P. Emerson, Jr. and Jane S. Bragdon, J. B. Lippincott Co., Philadelphia, 1959, pp. 857, $6.75.

The General Foods Kitchens Cookbook, Random House, New York, 1959, pp. 436, $4.95.

Principles of Nutrition, by Eva D. Wilson, Katherine H. Fisher and Mary E. Fuqua, John Wiley & Sons, New York, 1959, pp. 483, $5.95.

Ciba Foundation Study Group No. 1: Pain and Itch—Nervous Mechanisms, edited by G. E. W. Wolstenholme and Maeve O'Connor, Little, Brown & Co., Boston, 1959, pp. 120, $2.50.

Ciba Foundation Study Group No. 2: Steric Course of Microbiclogical Reactions, edited by G. E. W. Wolstenholme and Cecilia M. O'Connor, Little, Brown & Co., Boston, 1959, pp. 113, $2.50.

Human Nutrition and Dietetics, by Sir Stanley Davidson, A. P. Meiklejohn and R. Passmore, Williams & Wilkins, Baltimore, 1959, pp. 844, $15.00.

Klinische Radiologie des Magens und des Zwölffingerdarms, by Pierre Porcher, Hans-Ulrich Stössel and Paul Mainguet, Georg Thieme Verlag, Stuttgart, 1959, pp. 264, DM 72 ($17.75).

Metabolic Care of the Surgical Patient, by Francis D. Moore, W. B. Saunders Co., Philadelphia, 1959, pp. 1,011, $20.00.

Therapeutic Nutrition with Tube Feeding, by Morton D. Pareira, Charles C Thomas, Springfield, Ill., 1959, pp. 58, $3.75.

Abstracts of Current Literature

CHARLES R. SHUMAN, M.D., EDITOR

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MARTIN SILBERBERG, M.D., *St. Louis*
JANICE M. SMITH, PH.D., *Urbana*
GEOFFREY WALKER, M.B., *Oakland*
JOHN WATERLOW, M.D., *Kingston, Jamaica*

REDUCTION OF SERUM CHOLESTEROL LEVELS

The lowering of serum cholesterol has been achieved by means other than the substitution of unsaturated fatty acids in the diet for meat and dairy fats. One agent which exerts a consistent hypocholesterolemic action is nicotinic acid, the mechanism of which remains to be elucidated. A disagreeable side-effect of niacin therapy in large doses may be eliminated as described.

Effect of Salts of Nicotinic Acid on Serum Cholesterol. R. Altschul and A. Hoffer. *Brit. M. J.*, 2: 713, 1958.

It has been previously shown that large amounts of nicotinic acid taken orally can reduce the serum cholesterol levels in normal persons. Gastrointestinal symptoms are common with large doses of nicotinic acid and have been blamed on the acidity rather than on the compound itself.

In this experiment twelve subjects were given 1 gm. of nicotinic acid three times daily for two weeks; each gram was buffered with 0.6 gm. of sodium bicarbonate. There was a marked and statistically significant fall in serum cholesterol from about 200 mg./100 ml. to about 160 mg./100 ml., a similar fall to that found when unbuffered nicotinic acid is taken.

F. E. HYTTEN

The long-awaited demonstration of regression of established atheromatous lesions as well as reduction in serum and hepatic cholesterol levels has been accomplished by the administration of beta-sitosterol to animals. It would be of great interest to duplicate these observations in human subjects with atherosclerosis.

Effect of Beta-Sitosterol on Regression of Hypercholesterosis and Atherosclerosis in Chickens. E. R. Diller, B. L. Woods and O. A. Harvey. *Proc. Soc. Exper. Biol. & Med.*, 98: 813, 1958.

Cockerels about 200 days old were fed a broiler ration (7 per cent fat, 24 per cent protein, 3 per cent crude fibers) *ad libitum*. After eight weeks, cholesterol levels were determined, and for three weeks the birds were then given the basal diet supplemented with 5 per cent cottonseed oil and 2 per cent cholesterol. In some animals serum, liver and aortas were studied for cholesterol concentrations and atheromatous changes, respectively. The remaining animals were kept for an additional eight weeks on one of the following regimens: (1) basal diet supplemented with 1 per cent cholesterol; (2) basal diet supplemented with 1 per cent cholesterol and 4 per cent sitosterol; (3) basal diet supplemented with 4 per cent sitosterol; and (4) basal diet only. Both supplementation of the diet with beta-sitosterol or withdrawal of cholesterol from the diet reduced the elevation of the cholesterol concentration caused in serum, liver and aortas by cholesterol feeding, the tissue cholesterol concentration gradually approaching normal values. Sitosterol reduced the total cholesterol concentration of the liver below the level found after feeding the basal diet and caused regression of the atheromatous lesions in the aortas produced by cholesterol feeding. This regression paralleled the drop in cholesterol concentration in blood and liver.

M. SILBERBERG

Combined Beta-Sitosterol and Linoleic Acid Regimen for Cholesterol-Fed Rabbits. O. J. Pollak. *J. Gerontol.*, 13: 140, 1958.

One hundred ninety-eight male rabbits weighing about 3½ pounds were fed a stock diet of Purina rabbit chow plus 1 gm. cholesterol. Groups of animals received supplements of placebo, of beta-sitosterol in amounts of 1 to 7 gm. or of linoleic acid in amounts of 0.5 to 10 gm. alone or in varying combinations. The

animals were observed for two weeks. While total serum proteins, albumins and globulins remained unchanged, blood cholesterol was sevenfold increased after consumption of the cholesterol-enriched diet. Addition of sitosterol or linoleic acid inhibited or prevented the hypercholesteremia, the effective dose being 7 parts of sitosterol or 11 parts of linoleic acid to 1 part of ingested cholesterol. Combined administration of sitosterol and linoleic acid was correspondingly more effective than if given singly. It seems that the synergistic action of linoleic acid is due to a catalytic effect on sitosterol.

M. SILBERBERG

The lowering of serum cholesterol by reserpine has been attributed to a serotin-like effect. However, one would like to know the effect of reserpine administration upon weight curves, food intake, activity and other factors which may influence serum and tissue cholesterol.

Effects of Reserpine on Cholesterol Levels in Cholesterol-Fed Rabbits. C. Somoza. *Proc. Soc. Exper. Biol. & Med.*, 99: 347, 1958.

Male albino rabbits weighing about 2.8 kg. were fed a daily ration of 100 gm. of a stock diet containing 0.2 per cent cholesterol and were given saline injections thrice weekly. Another group received the same diet plus 0.04 mg./kg. reserpine three times weekly by intramuscular injections; a third group receiving the stock diet without supplements of cholesterol were also given injections of reserpine. The observations were extended over a period of six weeks. The cholesterol-fed rabbits had a mean of 357 mg. per cent total serum cholesterol as compared to 150 mg. per cent found in those treated with reserpine at the same time. Reserpine thus depressed the serum cholesterol elevation produced by cholesterol feeding; it also attenuated the lipid deposition in aorta, liver, spleen and adrenals as compared to that observed in animals fed the cholesterol diet, but not receiving reserpine. Some animals fed a cholesterol-free diet but treated with reserpine showed kidney lesions; these consisted of dilatation or collapse of the tubules with interstitial inflammation, changes resembling those seen after administration of serotin. It is thought possible that the hypocholesteremic effect of reserpine might be due to a serotin-like action.

M. SILBERBERG

Factors in the Hypocholesteremic Response of Patients Given a Brain Extract. R. J. Jones and T. F. Keough. *J. Lab. & Clin. Med.*, 52: 667, 1958.

An extract of brain was given to fifty ambulatory patients whose serum cholesterol had been measured at monthly intervals for six to eight months. The majority of these patients were on long term dicumarol therapy during the study and others were on low fat diets. A "statistically significant reduction of mean serum cholesterol occurred in 55 per cent of the group studied" during the period brain extract was administered. The authors suggest that this extract restricts intestinal absorption of cholesterol and other lipids.

Whatever the merits of the extract of brain, this study was not very well designed. Diet was not controlled, the dose of extract was not constant and subjects were probably taking a variety of other medications.

G. HOLLIFIELD

Species differences in responses to a potential hypocholesterolemic agent are manifested in the following study in which liver dysfunction was observed in human subjects but was absent in animals.

Effects of Δ^4-Cholestenone in Animals and in Man. D. Steinberg, D. S. Fredrickson and J. Avigan. *Proc. Soc. Exper. Biol. & Med.*, 97: 784, 1958.

Rats fed unlimited amounts of a cholesterol-free diet containing 1 per cent Δ^4-cholestenone, showed slowing of growth which pair feeding experiments showed to be due to reduced food intake, as well as gross adrenal cortical hyperplasia with reduction of ascorbic acid and cholesterol and increased dihydrocholesterol content. These effects were reduced but not abolished by adding 1 per cent cholesterol to the diet. Diets containing 1 per cent dihydrocholesterol had no effect on growth but caused definite adrenal hyperplasia. Diets containing 0.1 per cent Δ^4-cholestenone had little effect on either growth or adrenal size.

Studies with ^{14}C labeled Δ^4-cholestenone showed that at least 70 per cent of the sterol was absorbed from the gut in twenty-four hours and that about half the fecal ^{14}C was in the saponifiable fraction, suggesting that it had been excreted into and not merely not absorbed from the gut. Non-saponifiable ^{14}C labeled lipid was found chiefly in the liver, gut wall, adrenal and the serum.

Two hypercholesterolemic patients (diagnosis not recorded) were given 2.5 to 5 gm. Δ^4-cholestenone daily, with no effect on the serum cholesterol level but with a striking increase in the serum dihydrocholesterol level and some reduction of bromsulphalein excretion, without other evidence of liver damage. In contrast, rats fed doses of Δ^4-cholestenone about 10 times greater, on a body weight basis, showed a reduction of serum cholesterol of approximately 50 per cent, a relatively much less marked increase in serum dihydrocholesterol level and no histologic evidence of liver damage.

G. WALKER

Lowering of serum cholesterol in animals may be achieved also by removing bile from the intestinal tract by fistulas or by feeding saponin both of which interfere with absorption of exogenous and endogenous cholesterol.

Dietary Saponin and Plasma Cholesterol in the Chicken. P. Griminger and H. Fisher. *Proc. Soc. Exper. Biol. & Med.*, 99: 424, 1958.

Cross-bred male chicks one day old, cross-bred female chicks about three weeks of age, and one year old

white Leghorn roosters were fed (1) a basal diet low in protein (18 per cent), (2) the basal diet with supplements of (a) 0.3 per cent cholesterol, (b) 0.6 per cent saponin, (c) 0.6 per cent saponin plus 0.3 per cent cholesterol or (d) of protein. Feeding of the low-protein diet for twenty-four days to one day old animals increased the plasma cholesterol to 253 mg. per cent as compared to 154 mg. per cent in chicks fed additional protein. Supplements of cholesterol gave plasma cholesterol values of 348 mg. per cent, while saponin caused a reduction of blood cholesterol to 219 mg. per cent. A combination of cholesterol and saponin resulted in intermediate cholesterol levels of 295 mg. per cent. The same trend was noted in chickens kept on the various dietary regimen from three weeks or one year of age on. However, a comparison of food consumption data disclosed that while with advancing age the depressing effect of saponin of blood cholesterol became less conspicuous, the saponins exerted their effect in animals fed low protein diets as well as in those receiving additional proteins. Binding of saponin with cholesterol in the intestine may decrease the amount of cholesterol available for reabsorption from the intestinal tract.

M. SILBERBERG

Obligatory Function of a Continuous Flow of Normal Bile for Cholesterol Absorption. A. C. Ivy, R. Suzuki and C. R. Prasad. *Am. J. Physiol.*, 193: 521, 1958.

Cholesterol-4-C^{14} and cholesterol-4-C^{14} stearate are absorbed into the chyle of the rat to an average extent of approximately 44 per cent. They are not absorbed except in traces when the bile is diverted externally by means of a fistula. The artificial means used to date for restoring bile to the upper intestine have not resulted in the absorption of cholesterol or its stearate.

AUTHORS

Effect of Bile Acids and Other Factors on Cholesterol Uptake by Inverted Intestinal Sacs. A. L. Smith and C. R. Treadwell. *Am. J. Physiol.*, 195: 773, 1958.

Conditions for the use of inverted sacs of rat small intestine for quantitative studies of cholesterol uptake are described. The uptake of cholesterol by sacs did not require glucose in the incubation medium. Albumin aided cholesterol uptake but was not obligatory for this process. A binding of cholesterol to a cellular protein is proposed as the mechanism for the entrance of cholesterol into intestinal mucosal cells. Both conjugated and unconjugated bile acids inhibited cholesterol uptake possibly by blocking the binding sites of the protein responsible for cholesterol uptake. Commercial taurocholate and glycocholate contain an inhibitor of cholesterol uptake other than the bile acid. AUTHORS

METABOLISM OF UNESTERIFIED FATTY ACIDS

Most of the movement of fatty acids from fat depots to tissues using this substance as an energy source occurs as unesterified fatty acid (UFA) transported as an albumen-bound complex. Among the factors contributing to the release of UFA is that of glucose metabolism which, when increased, inhibits the mobilization of fat from tissue stores. Growth hormone as an anti-insulin factor effects an increase in UFA. Epinephrine exerts a similar action for which the presence of thyroid hormone may be required.

Effect of Growth Hormone on Plasma Unesterified Fatty Acid Levels of Hypophysectomised Rats. H. R. Engel, L. Hallman, S. Siegel and D. M. Bergenstal. *Proc. Soc. Exper. Biol. & Med.*, 98: 753, 1958.

The injection of growth hormone into hypophysectomized rats increased the plasma concentration of unesterified fatty acid but had no effect on the esterified fatty acid level.

This observation conforms with the findings of other workers, using intact animals, and contrasts with the effect of whole pituitary extracts which increase the concentrations of both esterified and non-esterified fatty acid.

G. WALKER

Mobilisation of Fatty Acids by Epinephrine in Normal and Hypophysectomised Rhesus Monkeys. H. M. Goodman and E. Knobil. *Proc. Soc. Exper. Biol. & Med.*, 100: 195, 1959.

Hypophysectomy prevented the increase of plasma non-esterified fatty acid concentration but had no effect on the increase of blood sugar after the injection of epinephrine in rhesus monkeys. Thyroid-stimulating hormone and tri-iodothyronine restored the non-esterified fatty acid response to epinephrine. Corticotrophin, hydrocortisone, prolactin and growth hormone failed to do so.

G. WALKER

In diabetes, the reduction in glucose metabolism sets the stage for mobilization of all classes of lipids. The level of UFA is raised in diabetic patients; this condition is partially corrected by insulin.

Fat Metabolism in Diabetes. V. P. Dole. *Bull. New York Acad. Med.*, 34: 21, 1958.

This short review article describes the impaired fat synthesis, increased fat mobilization and abnormal accumulation of fat in tissues found in patients with diabetes. Most of the discussion concerns the metabolism of plasma unesterified fatty acids which are now thought to be important in lipid transport. The material reviewed includes many recent papers.

M. W. BATES

The blood sugar lowering action of certain amino acids, such as leucine, arginine and glycine, has not been explained. The enhancement of glucose metabolism by arginine is a concept supported by the observation of UFA decrease in plasma of subjects receiving the amino acid.

Effect of Intravenous Amino Acids on Plasma Non-esterified Fatty Acids. S. A. Hashim and T. B. van Itallie. *Proc. Soc. Exper. Biol. & Med.*, 100: 576, 1959.

The intravenous infusion of a mixture of amino acids or of purified arginine was associated in normal adult

subjects with a fall of plasma non-esterified fatty acid and an increase of capillary-venous blood sugar difference. Both changes appeared promptly, were sustained during the infusion and disappeared within two hours of ending it. It is suggested that the fall of plasma non-esterified fatty acid in this experiment was due to increased glucose utilization. Insulin injection and carbohydrate feeding have a similar effect.

G. WALKER

Metabolism of Unesterified Fatty Acids (UFA) in Normal Dogs. P. S. Roheim and J. J. Spitzer. *Am. J. Physiol.*, 195: 288, 1958.

The source and disappearance of plasma UFA were studied in normal dogs by simultaneous lipid determinations in blood samples from the femoral artery, femoral vein, portal vein and hepatic vein. Marked and consistent venoarterial and frequent portal vein-arterial differences were found indicating UFA liberation in the leg and in the abdominal region, due to either lipolysis or mobilization from fat depots. Differences between UFA content of the portal and hepatic veins and of the portal vein and femoral artery were also consistent, showing uptake of UFA by the liver. The abdominal region also took up UFA frequently as indicated by arterial-portal differences. Similar changes were not detectable in triglycerides, total and free cholesterols and phospholipids.

AUTHORS

The albumen-UFA complex has been established as the major transport form of fatty acids. A rapid half-life of UFA has been demonstrated with C^{14}-labeling demonstrating the rapid oxidation of the fatty acids as an important energy yielding substrate.

The Metabolism of Albumin-Bound C^{14}-Labeled Unesterified Fatty Acids in Normal Human Subjects. D. S. Fredrickson and R. S. Gordon, Jr. *J. Clin. Invest.*, 37: 1504, 1958.

Twenty-one human subjects were studied. Injection of albumin-bound carboxyl-labeled palmitic, oleic and linoleic acids were made. Approximately 10 per cent of the labeled carboxyl carbon from palmitic and oleic acid was recovered as carbon dioxide in the first hour. Calculations suggest that a large proportion of the labeled acids recycle one or more times in the plasma before oxidation. This indicates that the flux of unesterified fatty acids from the plasma may be considerably greater than the utilization of these acids from energy. Recycling of linoleic acid was greater than for the other two. The authors present a general scheme for plasma unesterified fatty acid metabolism. This is a difficult paper to summarize but deserves reading in its entirety.

S. O. WAIFE

The Role of Unesterified Fatty Acid Transport in Chylomicron Metabolism. D. S. Fredrickson, D. L. McCollester and K. Ono. *J. Clin. Invest.*, 37: 1333, 1958.

Experiments with carbon-labeled palmitic acid were used in dogs to determine the importance of the blood unesterified fatty acid transport system in the metabolism of chylomicron triglyceride. A large fraction of the oxidized triglyceride fatty acid did not appear in the plasma as unesterified fatty acid. It appears reasonable to the authors that chylomicra represent a plasma vehicle for transport of fat which is directly available for oxidation.

It is particularly interesting that chylomicra appear to accumulate in the adipose tissue in the rat only after carbohydrate feeding, and release of fatty acids from adipose tissue is greatly decreased after carbohydrate feeding. In this study carbohydrate feeding decreased both the oxidation and amount of triglyceride fatty acids returned to the blood as unesterified fatty acids.

S. O. WAIFE

AMINO ACID FOOD FACTORS

Relatively short term feeding experiments on adult males have established the essential amino acids as valine, leucine, isoleucine, threonine, methionine, lysine, phenylalanine and tryptophane. For growing children, cystine and tyrosine may have to be added to the list. It is probable that in longer experimental periods histidine may prove to be essential. The availability of lysine in human diets has received considerable attention because of the low content of this factor in vegetable and cereal proteins. Lysine supplementation has been advocated to improve the biological value of these protein sources. In many areas the addition of milk to the diet will replace adequately the deficiency of lysine; however, in parts of the world with poor milk supplies other forms of supplementation should be provided.

Effect of Level of Protein Feeding upon Nutritional Value of Lysine-Fortified Bread Flour. R. S. Harris and D. A. Burress. *J. Nutrition*, 67: 549, 1959.

The authors believe that evaluation studies with plant proteins have been conducted at protein levels that are too low. Diets of mankind contain 12 to 15 per cent of total calories as protein. The average for the United States is nearly 15 per cent. It is noted also that rats are truly in a state of starvation when they are maintained on diets containing as little as 12 per cent protein. Since plant foods are relatively low in protein, it is necessary to concentrate the proteins in order to feed them at the desirable higher levels in experimental diets. In this study wheat flour (70 per cent extraction) was used for the 8 per cent protein feeding level, and wheat flour plus gluten for diets containing 15 per cent protein. The various diets were fortified with lysine from 0 to 2.48 gm. L-lysine hydrochloride per 100 gm. at the 8 per cent protein level, and 0 to 4.72 gm./100 gm. at the 15 per cent level. The higher lysine additions were calculated to be from five to eight times the amounts necessary to prevent it from being the most limiting amino acid in wheat flour. Egg albumin was used in the control diets at both levels. Another purpose of this study was to evaluate the possible effects of im-

balance when an excess of lysine is added to wheat flour protein.

Two hundred fifty-two male weanling rats were used. Feeding periods were fourteen and twenty-eight days. Water, alcohol-ether extracts, nitrogen and ash contents of pooled samples of liver and carcass were determined and recorded, as well as body weight gains. Protein efficiency was estimated from the gain in body weight per gram of protein consumed.

When fed at the 8 per cent protein level, lysine-fortified wheat protein was nutritionally inferior to egg albumin which was measured by increase in body weight, protein efficiency and carcass protein synthesis. At the 15 per cent level, lysine-fortified wheat protein was nutritionally equal to egg albumin as measured by increase in body weight and protein efficiency, but inferior in its ability to promote the synthesis of body protein. It is noted that the protein in the wheat flour diets contain a higher percentage of non-essential amino acids than egg albumin, which has a diluent effect on the efficiency of the protein; this protein also has a poorer balance of essential amino acids. The amount of carcass protein synthesized per gram of *essential* amino acids fed, whether as egg albumin or lysine-supplemented wheat flour at the 15 per cent level, was essentially the same. "Fatty livers" were observed in rats on the 8 per cent wheat protein diet, not at the 15 per cent protein level. Fatty livers were prevented by the addition of lysine to the 8 per cent wheat protein diets. A fivefold excess of lysine produced no toxic effects.

The nutritional evaluation of proteins intended for human use should be made by feeding experimental animals diets containing approximately 15 per cent of protein, which corresponds to the level of protein intake by man. FRANK E. RICE

Supplementation of Foods with Amino Acids. H. R. Rosenberg. *J. Agr. Food Chem.*, 7: 316, 1959.

Some new data are presented demonstrating the fact that lysine is the first limiting amino acid in the cereals on which most of the world depends for food—rice, corn and wheat. The author proposes and illustrates an interesting type of bar graph, from which can be read off the order in which other amino acids may be expected to be second limiting. Plotted are the amino acid requirements of the animal against the per cent of amino acid in the diet. FRANK E. RICE

Lysine Supplementation of a Breakfast Cereal and Milk Combination. R. Thiessen and G. H. Reussner. *J. Agr. Food Chem.*, 6: 368, 1958.

It has been demonstrated that the nutritional value of cereal foods derived from wheat has been improved by the addition of lysine. Since such foods are often eaten with milk, this study was undertaken to determine whether or not milk additives would serve equally well in supplying the lysine shortage. In all cases 8 gm. of sugar was added to each ounce of cereal (wheat flakes). Different mixtures were prepared by the addition of the following: (1) the equivalent of 4 ounces of milk in the form of dry whole milk, (2) 0.5 per cent lysine (DL-lysine hydrochloride), (3) a diet using casein as the protein was employed as reference. Vitamin supplements were added to all mixtures. Corn oil was added to those not containing whole milk. Food intakes were adjusted each day to equalize the protein intake.

The addition of lysine to the cereal brought about some response in growth, but it was not equal to that which resulted from the addition of milk. When lysine was added to the cereal-milk mixture, no significant increase was observed in growth or protein efficiency.

It was concluded that the relatively high level of lysine in the milk was sufficient to alleviate the lysine deficiency in the wheat flakes protein.

FRANK E. RICE

Evaluation of Lysine Supplementation of Infant Formulas Fed to Rats. L. S. Baur and L. J. Filer. *J. Nutrition*, 66: 545, 1958.

This investigation was undertaken to gain more precise knowledge on the possible loss of amino acids during the processing of milk-based foods, with particular reference to lysine. The rat was used, even though it is not an entirely satisfactory animal for nutritional experiments with diets of composition resembling human milk. Diarrhea proved to be a complicating factor in these experiments, as has similarly been reported by other investigators, due apparently to the inability of the rat to metabolize lactose when present at high levels. Diets used in these experiments were: (1) Similac powder, (2) Similac liquid, (3) an infant formula prepared from commercial evaporated milk with the addition of water, lactose, a mixture of corn, coconut and olive oils, and certain vitamins and minerals. These diets were calculated to contain 1.3 per cent L-lysine on a dry solid basis. Some of the test diets contained added lysine in varying amounts up to a level of as much as 200 per cent. In experiment I, the diets were begun without previous depletion. In experiment II, the rats were given a lysine-deficient ration for twelve days before beginning the feeding of the various milk-based diets.

Rats grew equally well whether fed evaporated milk, the dry milk formula, or the liquid milk formula. Growth was somewhat below optimum in all cases, however, due to inability of the weanling rat to adapt to the human-infant feeding formula. The addition of moderate amounts of lysine in experiment I (non-depleted animals) appeared to stimulate appetite; and as a result, the growth rate increased slightly. With lysine-depleted animals (experiment II), supplementation with lysine to the formulas was without effect on growth.

The authors conclude that "lysine addition to cow's milk protein furnishes no significant improvement in nitrogen efficiency." FRANK E. RICE

Effect of Dietary Supplementation with L-Lysine in Poorly Thriving Infants. A. J. Vignec and M. Z. Gasparik. *New York J. Med.*, 59: 1998, 1959.

This study was performed at the New York Foundling Hospital on infants approximately six to eight weeks of age at the onset. There were twenty-five infants with an average age of 7.1 weeks and 3.8 kg. body weight. Classification by anthropometric curves placed the infants in the third percentile; that is, 98 per cent of infants of similar age would weigh more. During the three-week control period, the diet was supplemented with a vitamin mixture. The diet consisted of evaporated milk and carbohydrate mixture providing a daily intake of 100 to 120 calories and 3.5 to 4 gm. of protein per kilo. During the second three weeks, in addition to the vitamins, supplemental lysine was added (250 mg. for infants under 10 pounds and 500 mg. for infants over this weight). During the final three weeks lysine was withdrawn.

Throughout the study the infants were eating well, and food consumption was not a problem. During the control period, the infants' average weight gain was 18.9 gm. per day. During the lysine test period it was 25.8, which is considered highly statistically significant, and in the postlysine period, the weight gain was 22.3 gm. per day. In a comparable control group of eighteen poorly thriving infants who did not receive supplemental lysine the rate of weight gain was not statistically significant in the first and second three-week periods. These data suggest a cause-and-effect relationship between the use of supplemental lysine and the increased rate of weight gain in the test group.

S. O. WAIFE

The maintenance of nutritive balance depends upon the provision of nutritional factors equivalent to or greater than the tissue requirements for specific substrates. The biologic availability of nutritive factors in the diet represents an important part of this equation. The chemical evaluation of a protein with respect to amino acid composition may fail to render a true measure of its biologic value. For this reason more complicated bioassay methods are frequently required.

Biological Availability of Essential Amino Acids to Human Subjects. II. Whole Egg, Milk and Cottage Cheese. J. H. Watts, L. K. Booker, J. W. McAfee, D. C. W. Graham and F. Jones. *J. Nutrition*, 67: 497, 1959.

The true biologic value of a protein depends not only on the amounts of the essential amino acids present but also upon their availability. Availability depends on several factors, among which are: (1) resistance to hydrolysis by digestive enzymes, (2) amino acid antagonism, (3) absorption rates of the individual amino acids and (4) in some cases solubility. The importance of evaluating availability of the amino acids in food proteins is therefore obvious. Eight healthy male students were employed. Egg protein was used as the reference protein. Five dietary periods were investigated, (1) a period of low nitrogen intake, (2) egg, (3) whole milk, (4) egg and milk, (5) cottage cheese. All diets in all periods except (1) provided essential amino acids above "safe intakes" as defined by Rose, except that in the cottage cheese and milk diets the sulfur-containing amino acid content was slightly low.

Detailed data are reported on each subject on each diet together with averages, for the following: nitrogen intakes, urinary and fecal nitrogen, nitrogen balances, and also the percentage availabilities of each of the eight essential amino acids, and cystine and tyrosine. Although the project was planned so that the experimental diets should be isonitrogenous as well as isocaloric, the final analyses of the diets as consumed showed some differences. Dietary nitrogen was somewhat lower during the milk intake and cottage cheese period, although the total amount of milk consumed (2 quarts/day) during the milk period was greater than average human consumption.

Availabilities of all amino acids were high for the most part in all diets. From a close study of the data, the following "tendencies" appeared. The egg-plus-milk diet gave somewhat higher availability percentages than either egg or milk alone. Milk alone was fractionally lower than egg protein in availability of amino acids. Availabilities of amino acids from the cottage cheese diet were higher than corresponding values in the milk diet, but lower, except for isoleucine, and lysine, than those in the egg-plus-milk diet. The authors attribute the slightly lower availability of amino acids during the milk period to what may have been a disturbing factor—the higher than usual volume consumed per day.

Urinary creatinine group averages varied by less than 0.1 gm. creatinine/day and were considered to be constant during all periods. FRANK E. RICE

Biological Availability of Essential Amino Acids to Human Subjects. I. Whole Egg, Pork Muscle and Peanut Butter. J. H. Watts, L. K. Booker, J. W. McAfee, E. G. Williams, W. G. Wright and F. Jones. *J. Nutrition*, 67: 483, 1959.

The authors state that this is the first time human subjects have been used in determining the biologic availability of the amino acids of food proteins. Pork muscle and peanut butter data are here reported; egg protein is used as the reference protein. Six healthy male students twenty-two to twenty-eight years of age were used. Each subject participated during four experimental periods: (1) low-nitrogen intake seven days, (2) egg period ten days, (3) pork period ten days and (4) peanut butter period ten days. The amounts of the amino acids in diets (2) and (3) were calculated to to be in excess of the safe intakes recommended by Rose (twice the minimal level required for a positive balance of nitrogen). The peanut butter diet (4) was low only in sulfur-containing amino acid content.

Numerous data are reported on urinary and fecal excretions of the eight essential amino acids and of

cystine and tyrosine. Percentage availability of each of the ten amino acids for each of the human subjects for the three foods together with averages, are calculated and reported in detail. Availabilities of amino acids were similar for the egg and pork diets. Peanut butter protein amino acids were consistently lower except for tyrosine. "Further research is needed to determine whether the difference observed in apparent availability of amino acids in these diets are due chiefly to difference in the rate of liberation of the amino acids from the protein moieties, to bacterial flora or to other factors."

FRANK E. RICE

Sequence in Which the Amino Acids of Casein Become Limiting for the Growth of the Rat. A. E. Harper. *J. Nutrition*, 67: 109, 1959.

It has been confirmed repeatedly that the adequacy of casein as a source of dietary protein for the rat is determined by its content of the sulfur-containing amino acids. Beyond that point there is little agreement as to the order in which the amino acids become limiting. While it is a simple matter to calculate the apparent sequence from knowledge of the amino acid composition of casein and of the requirements of the rat, it is important that the reliability of the sequence so calculated be checked by growth experiments with the animals.

This is a report on fourteen experiments in which approximately 600 rats were used. The basal diet contained 6 per cent casein. Either sucrose or dextrin was employed as the carbohydrate. Reported and discussed also are data on the influence of nitrogenous compounds other than the indispensable amino acids (glutamic acid, etc.), influence of type of carbohydrate, amino acid interrelationship, etc. It is concluded "that the sulfur-containing amino acids are most limiting; threonine next; then tryptophan, isoleucine, leucine, histidine, valine and phenylalanine all about equally limiting; lysine slightly less so than the preceding 6; and finally arginine, least limiting."

The finding that lysine is one of the least limiting of the amino acids is noted in relation to certain recent recommendations that this amino acid be added as a supplement to infant formulas prepared from cow's milk. The sequence determined experimentally obviously does not correspond with the calculated sequence. Three factors are believed to be responsible. (1) The amino acid analyses for casein may be in error. (2) All the amino acids in casein may not be readily available. (3) The estimated requirements for the rat may be high. The author recommends that the latter especially be reinvestigated. FRANK E. RICE

Recent evidence indicates that insulin increases the penetrance of amino acids into cells similar to its effect upon glucose transport through cellular membranes. Incorporation of amino acids into tissue proteir is an energy-requiring reaction for which oxidative processes and a source of hydrogen ions are required.

Incorporation of C^{14}-Amino Acids into Protein of Isolated Diaphragms: An Effect of Insulin Independent Glucose Entry. I. G. Wool and M. E. Krahl. *Am. J. Physiol.*, 196: 961, 1959.

The effect of glucose concentration and insulin on protein synthesis was investigated using the isolated rat diaphragm and C^{14}-labeled amino acids. When the donor rat has been well fed, the incorporation of histidine-2-C^{14}, lysine-2-C^{14}, phenylalanine-3-C^{14}, and alanine-1-C^{14} into muscle protein is not influenced by glucose concentration in the medium over the range 0 to 600 mg. per cent. Insulin leads to an increase in C^{14}-amino acid incorporation into muscle protein in the absence of glucose in the medium. This positive effect of insulin in the absence of added glucose is still observed when the diaphragms are pre-incubated for two hours prior to adding insulin. These findings suggest an effect of insulin on protein metabolism independent of its action in promoting glucose entry.

AUTHORS

Effects of Thyroxin on Amino Acid Incorporation into Protein. L. Sokoloff and S. Kaufman. *Science*, 129: 569, 1959.

Many clinical features of thyroid disease suggest a major role of thyroid hormone in protein metabolism, including growth and effects on nitrogen metabolism. Studies were undertaken to determine the effects of *in vivo* and *in vitro* thyroxin administration on the *in vitro* incorporation of DL-leucine-1-C^{14} into the proteins of rat liver homogenates. Leucine incorporation into the protein was augmented by the presence of thyroxin. In order to localize the source of this activity, mitochondria and microsomes were prepared, and the evidence indicates that most, if not all, of the increased activity is localized in the mitochondrial fraction.

These studies suggest that uncoupling of oxidative phosphorylation is not a physiologic action of thyroxin. Rather the evidence suggests that thyroxin stimulates energy-requiring processes, such as protein synthesis, and its characteristic acceleration of oxygen consumption is secondary to the increased demand.

S. O. WAIFE

Decreasing "Amino Acid Hunger" of Human Muscle with Age. H. N. Christensen, D. H. Thompson, S. Markel and M. Sidky. *Proc. Soc. Exper. Biol. & Med.*, 99: 780, 1958.

Injected C^{14}-labeled α-aminoisobutyric acid is not metabolized by man but is concentrated by muscle and probably by other tissues. Tissue uptake was measured directly by counting muscle removed at operation and indirectly by measuring residual plasma radioactivity and was found to be greater in children than in adults. Similar results have been obtained in rats. These findings support the concept that growing tissues have a greater avidity for amino acids than mature tissues.

G. WALKER

The excretion of amino acids is increased in the presence of severe liver disease, Fanconi's syndrome and in post-operative patients as well as in a number of other conditions including certain errors of metabolism, such as cystinuria. More information concerning amino acid excretion rates and factors influencing this function is vitally required.

The Urinary Excretion of Five Essential Amino Acids by Young Women. R. M. Leverton, F. S. Waddill and M. Skellenger. *J. Nutrition*, 67:19, 1959.

The increasing interest in the subject of amino aciduria in disease emphasizes the need for figures on the excretion of amino acids by normal subjects. The amino acids studied were threonine, valine, tryptophan, leucine and phenylalanine. All the essential amino acids, except the one being studied, were fed in the same amounts as they occur in 20 gm. of egg albumin. The one being studied was fed at various levels, both above and below the levels that have been suggested as a minimum requirement for young women. Data are presented for the following range of mg./day intakes: threonine, 0 to 765; valine, 375 to 650; tryptophan, 63 to 307; phenylalanine (with 900 mg. tyrosine), 120 to 220; and leucine, 95 to 1,860.

There was considerable variation among individuals at the same and at different levels of intake. Excretion values showed little relation to intake in the case of any of the amino acids, whether measured as total acid, or, as mg./1 gm. nitrogen, or, as mg./1 gm. creatinine excreted. Threonine and tryptophan did show some slight increase in excretion values when intakes were increased manyfold.

The authors do not average their figures on excretion of these amino acids by normal subjects.

FRANK E. RICE

ITEMS OF GENERAL INTEREST

Sex Difference in Rat of Ring A Reduction of Δ^4, 3-Ketosteroids In Vitro by Rat Liver. E. F. Yates, A. L. Herbst and J. Urquhart. *Endocrinology*, 63:887, 1958.

It has been found that liver homogenates from female rats reduce the alpha-beta ketone structure of certain steroids much faster than do homogenates obtained from male animals. In this investigation the degradation of a number of adrenocortical steroids as well as testosterone was studied, and marked sex difference was observed in all instances. The study shows that microsomes obtained from liver homogenates of female rats have a much higher concentration of the enzyme concerned with metabolic inactivation of the steroids than do liver microsomes obtained from male rats. Castration of male rats results in increased enzymatic inactivation of the steroidal alpha-beta unsaturated ketone structure whereas following the administration of testosterone enzymatic activity decreases. In view of the increased ability of female liver tissue to inactive adrenal steroid hormones, it is suggested that the secretion of these hormones by the adrenal of females must also be increased. It is hypothesized that this may be the explanation for the observation that the adrenals in female rats are larger than those in male rats.

A. B. EISENSTEIN

Function of the Postmenopausal Ovary, Comparison of Urinary Estrogen and Gonadotrophin Excretion and Response to Administration of FSH in Postmenopausal and Ovariectomized Women. C. A. Paulsen, R. B. Leach, H. Sandberg, S. Sheinfeld and W. O. Maddock. *J. Am. Geriatrics Soc.*, 6:803, 1958.

Using immature female rats in biologic assays for urinary estrogens and gonadotrophins, fifty-one postmenopausal and thirty castrate women were studied. The average urinary estrogen excretion of postmenopausal women was about twice that for castrate women. Urinary gonadotrophin values were on the average higher for ovariectomized women than for postmenopausal women. Urinary estrogen and gonadotrophin values remained constant with age in postmenopausal women. Two of six postmenopausal women given hog pituitary FSH showed increased urinary excretion of estrogen with relief of menopausal symptoms. Two similarly treated castrate women showed no response.

G. HOLLIFIELD

An Anabolic Study with Norethandrolone in Four Elderly Underweight Males. E. Woodford-Williams and D. Webster. *Brit. M. J.*, 2:1447, 1958.

Four men aged 56 to 80 were underweight after recent illnesses and persistently failed to gain weight on "an adequate diet of approximately 2,000 calories daily, containing 75 gm. of protein." They were otherwise apparently healthy.

After a control period on this diet, when it was established that body weight was constant and nitrogen in balance, body water was measured by a urea method and extracellular water by sodium thiosulphate. Thereafter treatment with norethandrolone, 50 mg. daily, was begun: (norethandrolone (17 α-ethyl-19-nortestosterone) is said to possess all the anabolic properties but only 6 per cent of the androgenic potency of testosterone propionate).

In from 22 to 42 days of treatment the four men gained from 1.7 to 4.0 kg. of body weight from 0.7 to 2.5 liters of body water and from -0.7 to 1.6 liters of extracellular water. There was a marked positive nitrogen balance. There were no unpleasant side-effects.

It seems reasonable to conclude that norethandrolone is a valuable treatment in cases of persistent failure to gain weight. It is probable that the tissue gained is solid rather than water but the use of methods with a big experimental error in only four subjects does not allow a detailed examination of this.

F. E. HYTTEN

Role of Food Consumption in the Mortality Response of Irradiated Rats Subjected to Prolonged Cold Exposure. B. D. Newsom and D. J. Kimeldorf. *Am. J. Physiol.*, 195: 271, 1958.

Prolonged exposure to a low ambient temperature increases the 30-day mortality rate of radiated rats. To determine if the increased mortality is due to a failure of the radiated rat to adapt to the higher metabolic demands of a cold environment, the food consumption of radiated (500 r, 600 r, 240 kv x-rays) animals was measured during 30 days of exposure at normal (23°C.) or lowered (6°C.) environmental temperature. The food consumption of radiated animals was increased by exposure to cold; however, this increase was not sufficient to maintain body weight at a level comparable to nonradiated animals. When the food consumption of nonradiated animals was limited to that of radiated animals during exposure to a 6°C. environment, the mortality observed among nonradiated animals was comparable to the increase in mortality induced by cold exposure of radiated animals. These results suggested that the increased mortality rate of radiated animals in a cold environment is related to an inadequate food intake. AUTHORS

Effect of Antibiotics on Growth of Lactose-Fed Rat. R. M. Tomarelli and F. W. Bernhart. *Proc. Soc. Exper. Biol. & Med.*, 99: 508, 1958.

Young rats weighing 80 gm. were fed a basal diet consisting of 24.2 per cent casein, 52 per cent lactose, 19.8 per cent fat, and 4 per cent salt mixture with vitamins. The effect of supplements of the following antibiotic and antibacterial agents on growth was studied: aureomycin HCl, bicillin, bacitracin, carbomycin B, polymixin B, chlortetracycline, sulfathalidine, and polymagma. Observations extended over a period of about six weeks showed that high-lactose feeding induced enlargement of the cecum probably due to increased bacterial growth, low body fat deposits and growth reduction to about two-thirds of the normal. Supplements of galactose and glucose prevented the changes in the intestine and restored normal growth. These results suggest that the disaccharides inhibit the overgrowth of the bacterial flora caused by high-lactose feeding. Of the substances tested only chlortetracycline, and to a lesser degree penicillin, increased the growth rate, even though these substances did not affect the low body fat deposition typical of the high-lactose feeding.

M. SILBERBERG

Timed Excretion of 5-Hydroxyindoleacetic Acid After Oral Administration of Bananas and 5-Hydroxytryptamine. C. E. Lewis. *Proc. Soc. Exper. Biol. & Med.*, 99: 523, 1958.

Healthy adult human males were given 300 to 500 gm. of banana pulp within less than one-half hour, about three hours after breakfast. A sixteen-hour sample of urine was taken prior to the ingestion of bananas. Subsequently urine was collected every 2 hours for a total of eight hours, and a second sixteen-hour sample was thus obtained. Determination for concentration of 5-hydroxytryptamine (5-HT) or serotinin and of 5-hydroxyindoleacetic acid (5-HiAA), the major metabolite of 5-HT were made. Absorption of 5-HT was quite rapid and complete. Conversion to and renal excretion of 5-HiAA took place without delay. The studies show, that in the evaluation of the significance of high levels of 5-HiAA in the urine, the dietary history of the patient is of importance. Samples of urine obtained more than eight hours after eating bananas should contain no or only small quantities of exogenous 5-HT. M. SILBERBERG

Orotic Acid: Nutritional Aspects. F. M. Chiancone, E. Ginoulhiac and L. Mainardi. *Acta Vitaminol.*, 12: 263, 1958.

A review of the literature and the results of personal investigations allow the conclusion that orotic acid, in conditions of inadequate feeding, can have a favorable action on body weight, reduce mortality (rat, chick), increase fertility (rat), and antagonize the effects of the diet on the development of endocrine glands (thymus, adrenals, uterus, ovaries of the rat). Orotic acid also has an influence on protein metabolism, where it increases utilization and synthesis, and plays a role in galactose metabolism. Furthermore, it shows a stimulating effect on the growth of immature children and a therapeutic value in some clinical syndromes, such as malnutrition in children, nutritional hemopathies and diseases of the liver. Some investigations concerning its influence on bacterial growth and its relations with competitive antagonists are reviewed. The importance of orotic acid in the field of nutritional studies is underlined. AUTHORS

Urinary Excretion of Beta Aminoisobutyric Acid in Irradiated Human Beings. J. R. Rubini, E. P. Cronkite, V. P. Bond and T. M. Fleidner. *Proc. Soc. Exper. Biol. Med.*, 100: 130, 1959.

Beta aminoisobutyric acid was detected in the urine of six subjects who were exposed to 57 to 276 r. total body radiation during an industrial accident; however, it was not found in the urine of two subjects exposed to 21 and 42 r., respectively. The urinary output of beta aminoisobutyric acid was roughly proportional to the radiation dosage and it is suggested that it reflected the severity of radiation-induced disturbance of desoxyribonucleic acid metabolism. G. WALKER

Autoimmunity in Thyroid Disease. M. J. Cline, H. A. Selenkow and M. S. Brooke. *New England J. Med.*, 260: 117, 1959.

Recent studies in animals and human subjects have further established the idea that autoimmune mechanisms may cause systemic disease. The authors report their investigation of the presence and persistence of thyroid antibodies in the serum samples of thirty-nine

persons with chronic thyroiditis and other disorders of the thyroid. The antigens were saline extracts of human thyroid glands, kidney, liver and lung, as well as purified human thyroglobulin. Stavitsky's modification of the Boyden hemagglutination test was used, in which antigens are coated on erythrocytes treated with tannic acid. Of sixteen patients with chronic lymphoid thyroiditis, fourteen had a significant antibody titer to extract of human thyroid gland or to human thyroglobulin, but not to extracts of other human tissues or to thyroid extracts of animals. Thyroid antibodies were found in six of twenty-three patients with other thyroid disorders, including acute and subacute thyroiditis and also after administration of radioactive iodine for treatment of hyperthyroidism. The authors postulate that any non-specific injury to the thyroid gland (surgical operation or administration of radioactive iodine) may release thyroglobulin into the circulation. Thyroglobulin acts as an antigen, increasing specific antibody production and subsequent damage to the thyroid gland and resulting in chronic lymphoid thyroiditis. The pathologic classifications of chronic thyroiditis represent "stages in a single process." Thyroglobulin antibodies were found in the gamma-globulin fraction of the serum proteins. Investigations are in progress to determine the time sequence of antibody formation after injury to the thyroid, whether surgical or by administration of I^{131}. E. COHEN

Thyroglobulin Antibodies in Multiple Thyroid Diseases. R. M. Blizzard, G. G. Hamivi, T. G. Skillman and W. E. Wheeler. *New England J. Med.*, 260: 112, 1959.

Thyroglobulin antibodies, as demonstrated by red-cell hemagglutination technic were found in sera of fifty-four of 161 patients with definite clinical and laboratory diagnosis of thyroid disease. Such antibodies were found in only eight of 219 subjects of a control population. Laboratory indices of thyroid disease included measurement of protein-bound iodine, uptake of I^{131}, basal metabolic rate and serum cholesterol. Thymol turbidity and serum globulin levels were obtained on some patients; biopsy of thyroid tissue was performed in some cases. When the authors submitted forty-eight positive sera to an agar-gel immunochemical assay method, only five were positive. This was considered indicative of the lesser sensitivity of agar-gel diffusion as a detection method for antibodies in contrast to the red-cell hemagglutination technic. A poor correlation was found to exist between the types of disease and the presence of antibodies. Many patients with thyrotoxicosis, spontaneous myxedema, non-toxic nodular goiter and chronic thyroiditis show such antibodies in their sera. However, the production of antibodies may be secondary to any stimulus of hyperplasia or "non-specific thyroid insult" that would release thyroglobulin from the thyroid follicle. Autodestructive phenomena are considered by the authors to be an etiologic factor in spontaneous myxedema and in Hashimoto's thyroiditis. They suggest that some cases of cretinism may be accounted for by such "autodestructive" processes, that may have some role in controlling thyrotoxicosis. E. COHEN

Thyroid Autoantibodies in the Families of Cretins. W. H. Beierwaltes, V. N. Dodson and A. H. Wheeler. *J. Clin. Endocrinol.*, 19: 179, 1959.

The hypothesis that thyroid autoantibody in the mother produces intrauterine fetal hypothyroidism was tested. Sera of fifty-six patients of twenty families containing cretins and of twenty-one subjects of twelve normal families of the same age were tested by red-cell-coated thyroid antigen. Positive reacting sera were tested by complement fixation and precipitin technics. Only the sera of three of twenty mothers of cretins gave positive serologic results that could be repeated, with no cross-reactions with human kidney or liver tissue antigens. The sera of cretins, and of their fathers or siblings were free of antibodies to thyroid antigen. None of the mothers of cretins whose sera were tested had any history of thyroid disease. In a control family, one mother gave a positive test. She had thyrotoxicosis treated with radioactive iodine six months prior to the test for "autoantibodies." The authors correctly conclude that the results of their study do not indicate that transfer of maternal thyroid autoantibody via placenta is responsible for congenital hypothyroidism. E. COHEN

Testicular Lesions Following Ingestion of DL-Ethionine Studied by a Quantitative Cytologic Method. G. M. Goldberg, A. Pfau and H. Ungar. *Am. J. Path.*, 35: 383, 1959.

Male albino rats weighing 120 gm. were fed Purina Laboratory chow and water ad libitum; another group was fed the stock diet supplemented with a 0.5 per cent solution of DL-ethionine. After twelve days the left testicle was removed and studied microscopically; after twenty-four days the animals were killed and the contralateral testicles studied in addition to liver and pancreas. The changes in the testes were graded according to the classification of Leblond and Clermont. In the rats receiving ethionine for twelve days, no progress of maturation was found beyond the "cap" phase spermatids, while the more immature cells of the seminiferous epithelium had remained intact. There were also giant cells found. Ethionine thus had destroyed all acrosome phase spermatids, all mature and immature spermatozoa and had arrested the maturation from cell "association stage" 8 to 9. No changes in the interstitial cells had occurred. After twenty-four days the injurious action of ethionine was much more obvious, all seminiferous epithelia had disappeared and only Sertoli cells persisted while the Leydig cells had undergone distinct hyperplasia. The liver and pancreas showed the characteristic lesions of ethionine poisoning.

M. SILBERBERG

The Role of Lipides in Electron Transport. III. Purification and Identification of a Cytochrome *C* Reductase Lipide "Cofactor." K. O. Donaldson, A. Nason, I. R. Lehman and A. Nickon. *J. Biol. Chem.*, 233: 566, 1958.

A highly purified lipid co-factor of cytochrome *C* reductase isolated from an n-hexane extract of bovine heart muscle was identified as glyceryl monostearate, -oleate, -palmitate by various chemical and physical technics. This co-factor was twenty times as active as alpha-tocopherol in reactivating isooctane-extracted cytochrome *C* reductase and was destroyed by acid or alkali, whereas tocopherol reactivation was unaffected by either treatment. Although the crude lipide extract from heart contained some vitamin E, the lipide purified by fractionation and charcoal treatment contained no detectable tocopherol. In a subsequent article, the authors show that this co-factor probably acts by liberating endogenous vitamin E in the enzyme to "active sites." M. K. HORWITT

Antigenicity of Steroid-Protein Conjugates. S. M. Beiser, B. F. Erlanger, F. J. Agate, Jr. and S. Lieberman. *Science*, 129: 564, 1959.

Five hormones: testosterone, cortisone, deoxycorticosterone, estrone and progesterone, were coupled to bovine serum albumin. Antisera were obtained from rabbits immunized with alum-precipitates of each of the steroid-protein conjugates. The evidence presented in this brief paper demonstrates that several steroid hormones may act as haptens when coupled to a protein and that antibodies with steroid specificity may be elicited. S. O. WAIFE

Orotic Acid and Hematopoiesis: Some Experimental and Clinical Observations. L. Bussi. *Acta vitaminol.*, 12: 301, 1958.

The results which have been obtained from investigations on the action of orotic acid *in vitro* and *in vivo* in experimental and clinical conditions of hematologic interest are presented. It appears that orotic acid can establish the normoblastic transformation in some patients suffering from macro-megaloblastic anemia. The importance of orotic acid as a necessary "substrate" for the synthesis of pyrimidines and thus of nucleic acids is underlined. When "substrates" of this kind are deficient, the action of vitamin B_{12} and of the folic acid-folinic acid system can be markedly reduced or even inefficient. AUTHOR

Nutritive and Metabolic Relations Between Orotic Acid and Vitamin B_{12}. R. Viviani and M. Marchetti. *Acta vitaminol.*, 12: 275, 1958.

Several investigations carried out in the Institute of Biological Chemistry, University of Bologna, have shown the existence of nutritive relations between orotic acid and vitamin B_{12} in the diet deficiency of the animal protein factor of casein; in fact, both compounds, vitamin B_{12} in a partial way and orotic acid in a more complete way, show a protective action against the effects of this deficiency. These relations have been further investigated in the metabolic field through the evaluation of the effect of orotic acid upon some biochemical changes which are present in the liver of rats with vitamin B_{12} deficiency, such as those of betaine-homocysteine-transmethylase, choline-oxydase, xanthine-oxydase, glucose-6-phosphatase, cytochrome-oxydase activities and of pantothenic acid and coenzyme A content. The results which have been obtained show that orotic acid cannot only promote the growth of rats with vitamin B_{12} deficiency, but also act similarly to vitamin B_{12} upon the metabolic changes induced by the same vitamin deficiency. These data throw some light on further relations between the two compounds. The conclusion is drawn that orotic acid appears to be involved in a series of metabolic reactions in which vitamin B_{12} plays a role. AUTHORS